# Epigenome dysregulation in cholangiocarcinoma

O'Rourke, Colm J; Munoz-Garrido, Patricia; Aguayo, Esmeralda L; Andersen, Jesper B

*Published in:*
BBA Molecular Basis of Disease

*DOI:*
10.1016/j.bbadis.2017.06.014

*Publication date:*
2018

*Document version*
Peer reviewed version

# EPIGENOME DYSREGULATION IN CHOLANGIOCARCINOMA

**Colm J. O'Rourke*, Patricia Munoz-Garrido*, Esmeralda L. Aguayo and Jesper B. Andersen**

Biotech Research & Innovation Centre (BRIC), Department of Health and Medical Sciences, University of Copenhagen, Ole Maaløes Vej 5, 2200, Copenhagen N, Denmark

*Authors contributed equally.

**Corresponding author:** Please address correspondence to Jesper B. Andersen, Andersen Group, BRIC at Email: jesper.andersen@bric.ku.dk, Phone: (+45) 35325834

**Disclosure:** The authors have nothing to disclose.

**Financial & competing interests' disclosure:** The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this chapter. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

**Grant support:** JBA is supported by grants from the Novo Nordisk Foundation, Danish Cancer Society and Danish Medical Research Council. CJO has a Marie Sklodowska-Curie postdoctoral fellowship, 2016-2018. PMU has a European Association for the Study of the Liver (EASL) Sheila Sherlock fellowship, 2016 and a Marie Sklodowska-Curie postdoctoral fellowship, 2017-2019.

**ABSTRACT**

Epigenomics is a fast-evolving field of research that has lately attracted considerable interest, mainly due to the reversibility of epigenetic marks. Clinically, among solid tumors, the field is still limited. In cholangiocarcinoma (CCA) it is well known that the epigenetic landscape is deregulated both during carcinogenesis and disease progression as a consequence of aberrant mechanisms leading to genome instability. In this article, we will briefly review the molecular alterations that have been described in the transformation of normal cholangiocytes into malignant derivatives, focusing on the role of non-coding RNA (ncRNA) interactions, DNA methylation, post-translational modifications (PTMs) of histones and chromatin remodeling complexes.

## 1. INTRODUCTION

Increasing incidence and mortality rates of cholangiocarcinoma (CCA) [1, 2] may potentially reflect an atypically high mutational burden shared by these malignancies in the biliary tract, which clinical intervention has thus far failed to identify [3]. It is therefore unanticipated that whole-exome [4] and whole-genome [5] sequencing approaches have revealed only intermediate global mutation rates in this cancer type [4, 6]. As such, the genetic alteration frequency is insufficient to entirely explain current clinical observations (such as the innate chemoresistance and rapid disease progression characteristic of CCA) or genomic observations (such as tumor subgroups characterized by grossly different transcriptomic profiles [4, 7, 8]). One substantial contributor to the missing oncogenic virulence could certainly involve epigenetics, heritable alterations in gene expression independent of DNA sequence changes, and epigenome dysregulation. This hypothesis is bolstered by the recurrent detection of mutations in epigenetic regulators (*IDH1/2*, *KMT2C*, *ARID1A*, *BAP1*), aberrations which likely have substantial (epi)genome-wide consequences.

Epigenetic information is transmitted trans-generationally through a variety of biochemical modifications and processes, including non-coding RNAs (ncRNAs), DNA and RNA modifications, histone post translational modifications (PTMs) and chromatin remodeling. The cholangiocyte epigenome constitutes a unique combinatorial state of these epigenetic marks, a minority of which are responsible for maintenance of biliary epithelial cell identity (and therefore, should remain constant) and a majority which fluctuate to control gene expression in response to endogenous and exogenous stimuli. Cholangiocarcinogenesis, like other neoplastic processes, is accompanied by gross epigenomic insult affecting almost all levels of epigenetic regulation. Such individual epigenetic alterations, or 'epimutations' [9], are becoming increasingly appreciated as potential pro-oncogenic lesions functionally synonymous to genetic mutations, though verification of true 'epi-driver' status remains elusive.

Comprehensive resolution of the origin(s) and perpetuation mechanism(s) of epimutations has not yet been achieved. One obvious contribution arises from classic genetic alterations (mutation and structural) of epigenetic enzymes, a phenomenon recurrently reported across all categories of epigenome regulators in CCA (**Figure 1**). However, given the widespread epigenomic insult in CCA that is emerging from the limited epigenome-wide association studies (EWAS) to date [10, 11], it is clear that other mechanisms (likely, epigenetic regulation of epigenetic regulators, rate-limiting enzymatic cofactors) also fuel the evolution of

CCA epigenomes. An enhanced understanding of these epimutational processes and their downstream consequences holds significant translational potential to improve both diagnostic and prognostic tools, as well as putative therapeutic strategies.

Accordingly, in this review we critically assess our current understanding of the processes that drive epigenome dysregulation in CCA, as well as their immediate and downstream biological consequences. Epimutations are broadly separated into ncRNA alterations, DNA methylation aberrations and chromatin perturbations. Additionally, we establish a rational framework to guide future design and execution of EWAS for CCA to optimize the potential for translational and clinical success.

## 2. NON-CODING RNAs IN CHOLANGIOCARCINOGENESIS

Non-coding RNAs (ncRNAs) are endogenous RNA molecules which are not translated into proteins and have emerged as key transcriptome regulators in many different cellular pathways and systems. NcRNAs are grouped into two broad subclasses according to their number of nucleotides (nt), small ncRNAs (100-200 nt) and long ncRNAs (>200 nt). Small ncRNAs mainly act as translational repressors and include microRNAs (miRs), piwiRNAs (piRNAs), small interfering RNAs (siRNAs) and small nucleolar RNAs (snoRNAs). On the other hand, long ncRNAs (lncRNAs) are generally involved in gene silencing. As such, this category of ncRNAs has lately emerged as new players in the malignant transformation of cholangiocytes [12], being able to act as oncogenes or tumor suppressor genes (TSGs) and, therefore, representing potentially valuable tools as predictive biomarkers or as therapeutic targets.

### 2.1 MicroRNA (miR) dysregulation in cholangiocarcinoma

MiRs are, so far, the best studied ncRNAs. Initially discovered in 1993 [13], these highly conserved, single-stranded 19-25 nt ncRNAs function as genomic rheostats that exert transcriptional control and impact translation by affecting initiation and mRNA destabilization of their multiple target genes [14]. Thus, miRs have a significant role in diverse fundamental cellular processes such as cell differentiation, proliferation, migration, cell cycle control and apoptosis. Through their capacity to regulate various genes, and consequentially multiple pathways, miRs are significant contributors to disease heterogeneity, a property which suggests translational potential for patient stratification, as well as confounding drug response [15]. Preliminary studies attempting to clarify the contributory pathogenic role of miRs in CCA have mainly focused on single candidate miRs. Accordingly, different studies have reported that miR-21 is upregulated in CCA patient tissue [16, 17], as well as in diverse CCA cell lines [18]. High levels of miR-21 have also been detected in serum of CCA patients [19]. Induced overexpression of miR-21 *in vitro* can lead to downregulation of the MMP inhibitor RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) subsequently increasing proliferation, invasion and migration [17] and tumor growth [20]; PTPN14 (protein tyrosine phosphatase non-receptor type 14), regulating invasion and metastasis[17]; and tumor suppressor PTEN, causing chemoresistance in CCA cell lines [18]. Moreover, deregulation of the miR-200 family (enclosing 5 members divided in two clusters, miR-200a, mir-200b and miR-429 in chromosome 1; miR-200c and miR-141 in chromosome 12) contributes to the early stages of metastasis by epithelial-to-mesenchymal (EMT) transition. [21]. Although various studies have shown that expression of miR-200

family members is downregulated in biliary tract cancers compared to normal tissues, leading to EMT activation and cancer cell invasion [22, 23], miR-141 and miR-200b were previously reported to be significantly overexpressed in malignant cholangiocytes and further contribute to tumor growth and gemcitabine resistance [18]. These confounding results reflect both their ability to act as oncomiRs or tumor suppressors, as well as suggesting that certain miRs can retain dual roles depending on the cellular context. Further, miRs like miR-34a [24], miR-204 [25, 26], miR-214 [27] and miR-221 [28] have also been reported to regulate EMT in CCA, thus representing potential therapeutic targets for preventing post-operative recurrence. Other miRs involved in CCA development and progression are summarized in **Table 1**.

The expression of miRs can be controlled at both the transcriptional and post-transcriptional level. Also, endogenous (hormones, cytokines) and exogenous (xenobiotics) compounds may affect miR regulation. Intrahepatic CCA (iCCA) has previously been classified into two different biological subtypes with markedly different outcomes (i.e., the `Proliferation´ and `Inflammation´ subsets) [8]. The main mediator of the latter tumor subset is the inflammation-associated cytokine Interleukin-6 (IL6), known to be overexpressed and to contribute to tumor growth in CCA. In this regard, IL-6-mediated hypermethylation of miR-370 leads to overexpression of the oncogene *MAP3K8* [29]. In addition, IL-6 has been shown to upregulate the expression of DNA methyltransferase 1 (DNMT1) and epigenetically alter the expression of *miR-148a* [30] and *miR-152* [30], both of which can bind and regulate DNMT1. The connection between miR and epigenetic modifications of genes has also been reported in extrahepatic perihilar CCA (pCCA), where miR-373 is downregulated and negatively regulates methyl-CpG-binding domain protein 2 (MBD2), which in turn hampers the methylation-mediated tumor suppressor *RASSF1A* [31, 32]. miR-200 family members (miR-200a, miR-200b and miR-429), which share a common promoter, have been found to be hypomethylated in CCA [33]. As a consequence, subsequent miR upregulation results in significant downregulation of the miR-target genes, including tumor suppressors *DLC1*, *FBXW7* and *CDH6* [33].

To date, limited large-scale miRNome studies are available in sufficiently well-characterized CCA patient cohorts, including subsequent patient-matched integrative '-omics' functional investigations. Palumbo *et al.* [34] recently performed a functional high-throughput miR mimic screen with a library covering 316 different miRs. The study was performed using the human extrahepatic bile duct CCA cell line (TFK1), and designed to elucidate miRs regulating CCA cell proliferation (reduced cell growth of 50% was considered as a positive hit). MiR-410 was the top suppressor of growth out of the 21 miRs identified to regulated CCA cell growth [34]. Concurrently, Lin and colleagues [35] used small RNA sequencing to determine the miR expression profiles in 24 human CCAs. In total, 3 members of a common miR cluster (let-7c, miR-99a and miR125b) were found to be downregulated in the tumors, which aberrant regulation further was associated with the activation of the IL-6/STAT3 signaling pathway, reinforcing the key role this signaling axis plays in cholangiocarcinogenesis [36].

### 2.3 Long non-coding RNAs (lncRNAs) in cholangiocarcinoma

Long non-coding RNAs (lncRNAs) are known to play important regulatory roles in development and progression of different types of cancer. The length of these non-protein-coding molecules (>200nt) enables them to form secondary or tertiary structures, and themselves function as RNA sequences. However, little is known about the role of lncRNAs and their downstream mechanisms in the pathogenesis

of cholangiocarcinoma (**Figure 2**). It has been reported that some lncRNAs play an important function in the regulation of inflammatory response pathways stimulated by viral and/or fluke infections, as well as oxidative stress [37]. This is the case of H19 and HULC, which in CCA are both activated by oxidative stress and target IL-6 and CXCR4 respectively, subsequently promoting cell migration and invasion [37]. Interestingly, the biological mechanism(s) underlying this event involves the `miR sponging´ phenomenon. A mechanism, in which lncRNAs possess the ability to affect the miR activity through direct binding and therefore impede miR binding to its usual target (miR-target gene). Thus, lncRNA (H19) has been shown to `sponge´ let-7a/let-7b, subsequently increasing the expression level of its target IL-6 [38]. Additionally, HULC can bind miR-372 and miR-373, which are suggested to target CXCR4. As such, overexpression of IL-6 and CXCR4 in CCA cells consequently results in a chronic inflammatory response, promoting progression of the tumor. This regulatory mechanism highlights the importance of the lncRNA-miR-mediated crosstalk. Another example of sponging involves the lncRNA MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1), miR-204 and its target CXCR4, which through this interaction promotes proliferation, invasion and migration of human pCCA [39]. Interestingly, the nuclear paraspeckle assembly transcript 1 (NEAT-1) lncRNA has recently been reported to have a pivotal role in modulating CCA drug sensitivity [40]. This study emphasized how reduced mRNA expression of the chromatin modulator *BAP1* (BRCA-1 associated protein-1) enhances sensitivity to gemcitabine in the CCA cell line (KMBC). Additionally, in an attempt to identify BAP1-regulated candidate lncRNAs with a role in controlling CCA drug response, an inverse correlation was observed between *BAP1* and NEAT-1 expression. This demonstrates that NEAT-1 acts as a functional downstream target of BAP1, and how their interplay may modulate drug responses. Nevertheless, the direct mechanism by which NEAT-1 modulates BAP1 control still remains unknown. As such, the BAP1 expression level varies substantial between different CCA cell lines. Recently, the mitochondrial enzyme encoded by *CPS1* and its lncRNA (CPS1-IT1) were shown to promote proliferation of iCCA cells [41], suggesting a putative role of this lncRNA as a potential diagnostic and prognostic biomarker in iCCA. Also, a recent study determined that the lncRNA (AFAP1-AS1) promotes growth and metastasis of CCA cells [42]. As such, AFAP1-AS1 deficient cells gave rise to smaller xenograft tumors compared to control. In addition, the study demonstrated an elevated expression level of AFAP1-AS1 found both in CCA tissues and cell lines compared to matched adjacent non-tumoral tissue as well as normal biliary epithelial cells, respectively.

High throughput methodologies have revealed an unexpectedly large number of abundant lncRNAs by far surpassing the miRNome. Furthermore, experimental evidence increasingly suggests the presence of network interactions (sponging) between lncRNA and microRNAs [43]. Transcriptomic profiling of 77 iCCAs and their adjacent non-malignant tissues was performed using lncRNA and mRNA microarrays [44]. This study defined more than 5100 lncRNAs and 6500 mRNAs as differentially expressed between paired tumor and adjacent noncancerous tissues. Further co-expression modules identified a total of 290 significant lncRNA-mRNA target gene pairs. Within this list, 6 gene pairs were previously reported to be associated with tumorigenesis in other cancers. Validation of these lncRNA-mRNA modules by qRT-PCR showed that four pairs (RNA42085-SULF1, RNA47504-KDM8, RNA58630-PCSK6 and RNA40057-CYP2D6) were positively correlated in iCCA tissues. As such, these pairs may have prognostic significance in CCA, i.e., patients with low levels of PCSK6 and CYP2D6 were found to have a reduced overall survival. Another recent analysis of the lncRNA landscape was performed in 34 iCCA patients, including their matched adjacent non-cancerous tissues and samples obtained from 4 healthy individuals [45]. Whereas, targeted qRT-PCR analyses were performed on all samples only 4 samples were used for lncRNA-mRNA expression profiles. In this study, a

total of 2716 lncRNAs and 883 mRNAs were found to be differentially expressed, with 142 putative lncRNA-mRNA pairs comprising co-modular signaling networks. Though current results are limited in scope, taken together, these studies reinforce the premise that the lncRNA landscape in CCA may contribute to disease pathogenesis and possibly drug resistance. Nevertheless, to attribute any concrete biological or clinical roles to lncRNAs or lncRNA-mRNA modules, larger and well characterized cohorts are needed.

## 3. DNA METHYLATION IN CHOLANGIOCARCINOGENESIS

### 3.1 DNA methylome dysregulation in cholangiocarcinoma

CpG dinucleotide methylation is by far the most prevalent DNA modification in the human genome. DNA methylation involves the *S*-adenosyl methionine (SAM)-dependent transfer of a methyl group onto cytosine to generate 5-methylcytosine (5mC). This fundamental epigenetic process is actively written by DNA methyltransferase enzymes (DNMTs) and erased by Ten-eleven translocation methylcytosine dioxygenases (TETs). Genomic distribution of CpG sites is non-random and can be categorized as CpG-rich regions or CpG islands (CGIs), CGI borders (CpG shores and shelves, 2kb and 4kb up- and downstream of CGIs, respectively) or CpG-depleted ('open sea'). These CpG-oriented domains co-localize with different regulatory elements to modulate transcription (promoters), facilitate long-range regulation (enhancers) and promote genomic stability (inter-genic elements, repetitive sequences). DNA methylation alterations are among the earliest molecular lesions to occur in tumorigenesis [46], displaying classic cancer hallmarks such as tumor suppressor promoter hypermethylation leading to transcriptional inactivation and, later in disease progression, global hypomethylation resulting in reactivation of endoparasitic sequences [47]. Characterization of the CCA methylome is still limited, though several classic epimutation mechanisms have been confirmed alongside some atypical features (**Figure 3**).

Given the functional diversity of presence or absence of CpG methylation sites at different regulatory regions, it is expected that modes of DNA methylation alteration are also non-uniform in CCA. Using a CGI-array (containing 237,000 probes), DNA hypomethylation events were found to be more common than hypermethylation (55.3% versus 44.7%) in 18 CCAs (mixed anatomical location), though hypermethylation events were more recurrent across patients (60% versus 40%) [11]. Suggestion of predominant hypomethylation conflicts with global 5mC quantification by LC-MS, which indicated comparable 5mC levels between a small sample set of CCA and surrounding normal [48]. This suggests the less CpG-rich regions of the genome, not covered by the CGI-array, may shift towards more methylated states or at least remain consistent in disease. Notably, 5-hydroxymethylcytosine (5hmC), the immediate downstream metabolite of 5mC during DNA demethylation, is significantly downregulated in iCCA, and is an indicator of poor prognosis [49]. Similar inter-chromosomal epimutational trends were observed with 94.8% X chromosome alterations comprising loss of methylation events, in comparison to predominant autosome hypermethylation. Evidence for sub-chromosomal regional differences has also been demonstrated, epitomized by the distal 30 megabase (Mb) region of chromosome 1. This region is commonly deleted in different cancers and contains many tumor suppressor genes. Intriguingly, gene bodies within this region in CCA are largely hypomethylated, suggesting substantial (though mechanistically unclear) transcriptional dysregulation.

Like other cancers, the most commonly described epimutations in CCA comprise promoter hypermethylation events of genes with tumor suppressor function. Binomial distribution of epigenome-wide hypermethylation events certainly suggest that these epimutations are not random but directed in CCA [11]. Epigenomic analysis of 18 CCA patients uncovered 65 distinct WNT pathway genes to harbor epimutations, predominantly targeting promoter and alternative promoter sites. Follow-up immunohistochemistry in a larger independent sample set confirmed significant transcriptional consequences of these epigenetic alterations for the WNT pathway, though such genes could be rescued *in vitro* by treatment with hypomethylating agent, 5-aza-2'-deoxycytidine [11]. In contrast, DNA hypomethylation is less well characterized in CCA. IMP3, a fetal oncoprotein that is restricted to expression during development, was found to be reactivated in iCCA [50]. Such transcriptional activation was mediated by gross promoter hypomethylation, highly recurrent (high immunohistochemical staining in 82% of patients) and independently associated with multiple adverse prognostic parameters (tumor volume, grade, metastasis and overall survival). DNA methylome alterations are not restricted to protein-coding genes and frequently target miRs in CCA [33], as discussed in the ncRNA section this review. Many of the epimutations described in CCA are not disease-specific and are found in other types of gastrointestinal cancers (including gastric, pancreatic and colorectal) [51]. Certain epimutations synergize with co-occurring mutations as indicated by the adverse outcome of *TP53* mutant CCA patients also presenting aberrant promoter hypermethylation of genes (*DAPK*, *p14*, *ASC*) involved in the mitochondrial apoptosis pathway [52]. Furthermore, certain loci are targeted by diverse perturbation mechanisms (mutational, epimutational or structural rearrangement) such as *RUNX3* promoter hypermethylation as well as 1p36.1 loss (containing *RUNX3*) in CCA, indicating universal tumor advantage by any perturbatory mechanism [53]. Integrating target gene data from different mechanisms may provide a novel method to in the future identify `true´ disease drivers, as well as likely increase the percentage of patients that may benefit from subsequent pathway-directed therapy.

Epigenetic modifications significantly govern differentiation states and, thus, cellular identity. It therefore logically follows that epimutations should target genes involved in differentiation during cholangiocarcinogenesis. Indeed, analysis of differentially methylated sites in CCA patient tissues has indicated an enrichment of epimutations in Polycomb Repressive Complex 2 (PRC2)-target genes [10]. One study remarkably found that 10% (97 out of 970) of aberrantly methylated CGIs mapped to *HOX* genes [54], further confirming preferential targeting of developmentally important loci. Focusing on the hepatobiliary system, *SOX17* is a highly specific marker of biliary differentiation and has emerged as a potent tumor suppressor in CCA [55]. Using a morphogen-driven *in vitro* differentiation system (from induced pluripotent stem cells to hepatic progenitors to induced cholangiocytes), SOX17 was specifically identified as a factor induced exclusively in the progenitor to differentiated transition. *In vitro* knockdown of *SOX17* in normal cholangiocytes was sufficient to induce loss of biliary marker (CK7, CK19) expression, as well as increase proliferation and cancer-associated gene expression profiles, whereas overexpression in CCA cell lines had inversely complimentary effects. *In vivo, SOX17* overexpression effectively inhibited growth of pre-existing tumors and completely impaired tumor grafting when administered simultaneously. Mechanistically, WNT3A was elucidated to downregulate SOX17 in a DNMT1-dependent manner, mirroring *SOX17* downregulation by promoter methylation in CCA patients. These findings highlight a unique case of tumor suppressive function that is tightly linked to biliary differentiation and is modulated by epimutations in cancer. Given the diversity of hypothesized cells of origin in CCA [56], it is likely that other differentiation-

associated epimutations exist and that these may vary between CCA patients arising from different cellular origins.

Akin to mutations, epimutations also appear to display temporal specificity. For example, promoter hypermethylation of *p16* has been well described in CCA but has also been detected in 45.6% primary sclerosing cholangitis (PSC) samples, a well-known predisposing risk factor for development of CCA, without any signs of malignant onset [57]. Similarly, this locus was also found to be hypermethylated in liver intraductal papillary neoplasia (IPN), a precursor lesion to CCA, and inversely correlated with gene expression [58]. Hepatitis C virus (HCV) core protein is capable of upregulating DNMT1, leading to suppression of miR-124 and induction of oncogenic SMYD3, linking early infection with pro-oncogenic effects [59]. In a series of matched extrahepatic CCA, biliary intraepithelial neoplasia (BiIN) and normal cystic ducts, 4 cancer-associated promoter hypermethylation events (*TMEFF2*, *HOXA1*, *NEUROG1*, *RUNX3*) were detected in pre-malignant lesions and retained in tumors, suggesting clonality of these epimutations [60]. Repetitive element analysis in these samples also revealed progressive hypomethylation of *SAT2* repeats from normal to BiIN and from BiIN to CCA, but *LINE1* hypomethylation exclusively occurred in BiIN to CCA transition. These findings begin to emphasize the early evolutionary dynamics of epimutations during cholangiocarcinogenesis. Little is known of these processes at the opposite end of the disease spectrum, however, during the transition from primary CCA to metastatic CCA. Promoter hypermethylation of *ITGA4* has been tentatively suggested as such, given its methylation status in 100% patients with lymph node metastasis in a modest study population [61]. Further clarification of the epimutational contribution to CCA dissemination is required given metastasis is the actual cause of death in >90% of cancer patients.

### 3.2 Origin(s) of DNA methylation alterations in cholangiocarcinoma

It is evident, even given the limited EWAS in CCA, that the DNA methylome undergoes widespread dysregulation with concomitant transcriptome destabilization. What lacks clarification, however, is the molecular origin(s) of these epigenomic insults. Two plausible scenarios currently exist: genetic (mutation and structural) alterations of DNA methylome regulators or epigenetic alterations of such epigenetic regulators.

Intrahepatic CCA is different among epithelial cancers in that approximately 10% of patients present tumors with mutations in *Isocitrate Dehydrogenase* (*IDH*) enzymes, *IDH1* and *IDH2* (otherwise mutated in tumors of mesenchymal and neuronal origins) [62]. Normally, IDH1 and IDH2 catalyze the conversion of isocitrate to α-ketoglutarate (α-KG) in the cytoplasm and mitochondria, respectively. When mutated, typically at hotspots R132 (*IDH1*) and R172 (*IDH2*), these enzymes acquire neomorphic activity and produce 2-hydroxyglutarate (2-HG). This oncometabolite severely impairs the normal function of multiple pathways, including DNA demethylation as mediated by TET2 [63]. The effects of these mutations are so pronounced in patient tumors that The Cancer Genome Atlas (TCGA) cholangiocarcinoma network has identified *IDH* mutant tumors as an independent subtype of biliary cancer [4]. *IDH* mutants have higher 5mC levels and lower 5hmC levels, indicative of DNA demethylation pathway dysfunction. Consequently, *IDH* mutant tumors are grossly hypermethylated in comparison to wild type tumors with DNA methylation alterations significantly enriched at promoters, depleted at gene bodies and predominantly targeting other epigenetic regulators [62]. This is further corroborated by observations of genes involved in chromatin regulation

being significantly downregulated in IDH mutant CCA [4], indicating a potential epigenomic 'snowball effect' between mutant IDH (functioning as an oncogene) and downregulated chromatin modifiers (functioning as tumor suppressors). Remarkably, however, IDH tumors did not on average have the greatest amount of DNA methylations alterations, re-emphasizing the unclear but significant contribution of other biomolecules outside DNA methylation pathways.

Since DNA methylation profiles are actively modulated in response to exogenous stimuli, it is logical to assume inter-cellular signaling mechanisms may direct methylome alterations. Accordingly, the inflammatory mediator IL-6 (which is overexpressed in CCA patients) and its signaling axis came to attention due to its capacity to attenuate the hypomethylating and subsequent cellular effects of 5-aza-2'-deoxycytidine treatment *in vitro [64]*. Epigenetic mechanisms sustain IL6 signaling in biliary tumors through promoter hypermethylation of its negative regulator *SOCS3*, thereby inhibiting the feedback loop [65]. Persistent IL-6 signaling in turn has pleiotropic effects including upregulation of cancer associated pathways [64], hypermethylation and downregulation of tumor suppressor genes [30], hypermethylation and downregulation of miRs (miR-148a, -152) [29] and, intriguingly, upregulation of *DNMT1* [29, 30]. Subsequently, *DNMT1* upregulation was shown to be due to IL6-associated downregulation of miRs that target the 3'-UTR of DNMT1 through an elusive mechanism [30]. This is another exemplar case of epigenetic snowball effects in CCA, whereby epigenetically-perpetuated IL-6 signaling (via *SOCS3* hypermethylation) downregulates epigenetic regulators (miRs) leading to upregulation of the epigenetic regulator DNMT1 and subsequent epigenetic modulation of tumor suppressor genes. Anecdotally, DICER also appears to be able to direct DNA methylation (specifically *SFRP1* promoter hypermethylation) through translocation to the nucleus, though the specific mechanism behind this is unknown [66].

### 3.3 Translational potential of the cholangiocarcinoma DNA methylome

Characterization of DNA methylation alterations in CCA to date has revealed that these events are recurrent and occur early during disease manifestation (including under predisposing conditions such as PSC and precursor lesions such as BiIN). These properties together indicate DNA methylation could be a useful biomarker tool for the early detection of CCA, arguably one of the biggest issues in the management of this disease given the low resectability rates (10-30%) of patients at time of diagnosis. *In vitro,* pharmacological rescue experiments using 5-aza-2'-deoxycytidine has led to the identification of a candidate 4-gene biomarker panel (*SFRP1*, *DCLK1*, *CDO1*, *ZSCAN18*) [67]. Targeted methylation analysis of these promoters correctly diagnosed tumor from normal controls with an impressive sensitivity of 87% and a specificity of 100%. These findings support the relevance of *in vitro* culture systems to understand molecular changes in patients, as well as highlight the predictive potential of epimutations in fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) samples. Nonetheless, biopsies taken at biliary tract are highly invasive and non-invasive tests (liquid biopsies) would be preferred. While tissue-guided selection of a 2-gene panel (*SHOX2*, *SEPT9*) did correctly identify some tumors when quantified in plasma [68], its performance was significantly poorer than in tissue, suggesting that these two biomaterials are not directly comparable from a DNA methylation perspective. Rather, preliminary data suggests bile may be a more relevant biofluid to question. Biliary brush cytology is currently used to aid diagnosis of CCA but possesses several limitations, including difficulty in discriminating atypical reactive epithelia from true neoplasia, low cell recovery and cellular disintegration. By initially testing in tissue samples, Andersen and colleagues

identified an optimal 4-gene panel (*CDO1*, *CNRIP1*, *SEPT9*, *VIM*) for targeted methylation analysis in bile samples [69]. To compare the diagnostic ability of this novel method against routine brush cytology, DNA methylation analysis achieved sensitivities of 73% and 91% in both test and validation cohorts, whereas cytology achieved 58% and 63% (specificity was comparable across diagnostic tools). As such, DNA methylation-based biomarkers may serve useful when the liquid biopsy is biofluid (e.g., bile) in close proximity to the hepatobiliary system.

Similarly, DNA methylation alterations are attractive therapeutic targets since, unlike mutations, they are reversible. Debate still exists as to the existence of 'epi-drivers' [70], epigenetic alterations upon which tumors have developed a functional dependence and which function synonymous to classic mutational drivers. Nonetheless, traditional use of 5-aza-2'-deoxycytidine as a pharmacological rescue agent across many DNA methylation studies confirms the *in vitro* efficacy of such agents. Zebularine, a second-generation nucleoside analog, competitively binds DNMTs, leading to an overall reduction in DNMT levels, genome hypomethylation and active induction of apoptosis in CCA [71]. DNMTs are overexpressed in CCA patients, though the extent to which is highly variable, indicating that a responder signature may be required to identify patient subgroups that could benefit from DNA hypomethylating agents [72]. However, the relatively poor solid tissue bioavailability of these compounds alongside peak cytosine deaminase (a rate limiter of demethylating agents) concentrations within the liver brings the therapeutic potential of these compounds into question.

## 4. CHROMATIN DYNAMICS IN CHOLANGIOCARCINOGENESIS

The fundamental unit of chromatin organization, the nucleosome, is comprised of a histone octameric complex around which 146bp DNA is spooled. Through a variety of histone post translational modifications (PTMs) added and removed by specialized epigenetic enzymes, nuclear compaction is regulated to direct gene expression profiles, facilitate DNA repair and promote genome stability. Analogous to ncRNA and DNA methylation epimutations, histone PTMs also become dysregulated in cancer. Of the many PTMs identified, only two biochemical modifications (histone acetylation, histone methylation) have been evaluated in CCA across a limited number of studies.

Histone acetylation is written by histone acetyl transferase (HAT) enzymes and erased by histone deacetylase (HDAC) enzymes. Addition of acetyl groups onto the histone tail is a pro-euchromatin event, altering the tail charge from positive to neutral and promoting loosening of the nucleosomes, while removal results in nucleosome compaction (a pro-heterochromatin event). A variety of HDACs have been uncovered as overexpressed in CCA, including associations with adverse prognosis [73, 74]. Unsurprisingly, the effects of aberrant HDAC expression appear to be quite diverse. One of the characteristic features of malignant transformation of cholangiocytes involves the shortening and/or loss of ciliary appendages. This loss could be driven *in vitro* by forced overexpression of HDAC6 (also resulting in increased proliferation and anchorage-independent growth) and subsequently rescued *in vivo* through treatment with the HDAC inhibitor (HDACi) tubastatin [75]. *SPRR2A*, a gene involved in maintenance of epithelial barriers and wound repair, was found to be elevated in a single CCA cell line. This event resulted in deacetylation of P53 by p300 acetylation and increased HDAC1 expression, providing a histone-based link for attenuation of normal TP53 signaling [76]. HDAC4 was identified as overexpressed in CCA through downregulation of its negative

regulator miR-29a facilitated by TGF-β1 signaling, resulting in increased metastatic characteristics *in vitro* [77]. A variety of compounds with HDACi activity, in particular Valproic Acid (VPA) and Vorinostat, have shown success in inhibiting CCA growth alone and in combination with chemotherapeutics *in vitro* [78, 79] and *in vivo* [80]. The downstream effects of HDACi treatment underpinning therapeutic responses remain poorly characterized, though it is in part mediated by TACC3 downregulation [74]. Nonetheless, in spite of such lacking molecular clarity, VPA appeared to display anti-neoplastic activity with manageable safety profiles in a small mixed cohort of pancreato-biliary patients (including CCA) [81] and Vorinostat *in vivo* effects appeared to be enhanced through delivery with nanoparticles [82]. A prospective phase 1b clinical trial (NCT02856568) will test the effects of treatment with Ricolinostat (HDAC6 inhibitor) in combination with standard gemcitabine and cisplatin in unresectable and metastatic CCA patients.

Unlike histone acetylation, the effects of histone methylation are varied dependent on the target amino acid. EZH2, the only histone methylation enzyme analyzed in CCA to date, is a histone methyltransferase specifically catalyzing the methylation of histone 3 at lysine 27 (H3K27me) mark. Acting from within the Polycomb Repressive Complex 2 (PRC2), H3K27me is associated with transcriptional repression. EZH2 is overexpressed in CCA and associated with poor prognosis in both intrahepatic and extrahepatic CCA [83]. Inhibition of EZH2, either through siRNA-mediated knockdown or the compound DZNep, substantially impairs tumor cellular properties *in vitro* (hyper-proliferation, evasion of apoptosis, efficient cell cycling), synergizes with gemcitabine treatment [83, 84] and slows tumor growth *in vivo*, in part through rescue of *RUNX3* expression [85].

Higher order regulation of nucleosomes and chromatin dynamics has additionally been implicated in CCA pathogenesis. Whole-exome and whole-genome sequencing has uncovered recurrent, frequently inactivating mutations in chromatin remodelers such as *ARID1A*, *ARID1B*, *BAP1* and *PBRM1* [86-88]. Chromatin modifiers are also targeted by alternative perturbation mechanisms, such as promoter hypermethylation and transcriptional silencing of *ARID1A* in *IDH* mutant tumors [4]. The functional implications of this impairment of chromatin remodeling in cancer cells require substantial clarification, though they do appear to act broadly as tumor suppressors (**Figure 4**). Indeed, functional testing through hepatocyte-targeted CRISPR screening *in vivo* confirmed positive selection of *ARID1A* mutations in cancer cells [89]. Specifically, ARID1A is a member of the SWI/SNF chromatin remodeling complex. ARID1A expression becomes lost in CCA, though its expression was still detected in pre-malignant lesions [90]. Similarly, another SWI/SNF complex member, PBRM1, displayed comparable patterns with normal expression in precursor lesions before becoming lost in CCA [91]. These findings indicate loss of expression of SWI/SNF members is a late event in cholangiocarcinogenesis and may underpin acquisition of the invasive cancer phenotype.

## 5. EXPERIMENTAL DESIGN & THE FUTURE OF CCA EWAS

Development of EWAS, particularly in oncology, has led to the retroactive identification of fundamental pitfalls in experimental approaches to identify true epimutational associations with a given phenotype [92, 93]. It is perhaps, therefore, relatively opportunistic that CCA epigenomics remains in its infancy and that careful study design may prospectively maximize the likelihood for detection of true disease-associated epigenetic alterations. Such issues identified to date include careful patient stratification, tissue

deconstruction of cell types and inference of association and/or causation (whether direct or reverse) (**Figure 5**).

Common population variables have been shown to influence DNA methylation profiles (and plausibly may affect other epigenome modifications). Unlike genetic information, DNA methylation profiles are temporal and have been proven to flux over time. Though CCA is typically a disease associated with advanced age, efforts should be made to design study populations with matched age profiles. Similarly, study populations should be controlled for ethnicity and disease etiology (e.g. HCV infection, alcohol exposure, fluke parasitism) as potential confounders, especially given the findings of alcohol- and HCV-associated DNA methylation signatures in hepatocellular carcinoma [94]. Gender distribution should also be considered. While sex chromosomes are typically excluded from DNA methylation analysis of mixed-sex disease (due to female X chromosome inactivation), many cancer-relevant genes are located on the X chromosome. Of note, Goeppert and colleagues identified gross DNA hypomethylation of the X chromosome in CCA that was demonstrated to be statistically independent of technical issues or sampling bias [11]. This suggests that sex chromosomes should not automatically be discredited in future CCA EWAS. Also, longitudinal sampling and epigenetic characterization should be pursued to characterize intra-individual epigenome evolution in response to disease progression and clinical intervention.

Given the intimate link between epigenomes and cellular identity, perhaps the biggest challenge in EWAS to date is the stratification of epigenetic signals based on their cellular origins and identification of biologically-relevant comparison points. CCA epigenetic studies have typically, like most other cancers, employed surrounding normal as the control to tumor. This has three primary limitations: (1) the majority of 'surrounding normal' tissues are cirrhotic and, therefore, will not have truly normal epigenomes; (2) even if surrounding tissue appears histologically normal, extensive field cancerization mediated by epigenetic field defects are common; and (3) cell subpopulation composition between tumor and control may significantly differ leading to spurious epimutation results, in particular in comparison of iCCA with its predominantly hepatocyte-derived surrounding normal liver tissue. Potential solutions to some of these issues include the use of appropriate donor tissues comprising truly normal cholangiocytes (potentially introducing inter-individual variability as a confounder) or extensive laser microdissection of samples. Alternatively, cellular deconvolution strategies should be incorporated into CCA EWAS. While this may be computed purely using statistical algorithms ('reference-free') [95], future establishment of reference cholangiocyte epigenomes ('reference-based'), alongside epigenomes of co-occurring cells (hepatocytes, fibroblasts, endothelial, immune), is highly warranted to accurately optimize these analyses to the hepatobiliary system.

Definitively proving the causative or consequential role of high confidence epimutations is notoriously difficult. Estimates of the extent of DNA methylation alterations driven by SNPs range from 22-80% [93], indicating a need for tight mutational controls in epigenomic studies. Similarly, discriminating epigenetic-driven transcriptomic changes from transcriptome-driven epigenetic changes supports matched epigenome and transcriptome profiling experiments. Such 'cross-omics' analysis was recently generated by the TCGA, highlighting *IDH* mutant-associated *ARID1A* promoter hypermethylation and subsequent transcriptional silencing [4]. Similar difficulties also exist at the phenotypic level in establishing whether epimutations are causative or a consequence of CCA. While certain epimutations have been tentatively proffered as 'epi-drivers' in liver cancer purely on the basis of recurrence, these are insufficient to address the

causation/consequence debate. Rather, functional epigenetics experiments involving the targeted modulation of epimutations followed by their rescue are required, technologies which are only in the early stages of development [96-98].

## 6. CONCLUSION

To date, CCA remains largely understudied from an epigenetics-perspective, likely due in part to its status as a rare orphan disease. Nonetheless, its unique epigenetically-orientated mutational profile indicates biliary tract malignancies may comprise ideal solid tumors to benefit from epigenetic biomarkers and, potentially, epigenome-targeted therapies enabled through continued CCA epigenome characterization. Many epigenetic modifications (for example, RNA modification or chromosome conformation states) have yet to be investigated and those which have (ncRNA, DNA methylation, histone PTMs, chromatin remodeling) require expansion and refinement to (epi)genome-wide scales, facilitated by universal EWAS guidelines and standards. Only then may epigenomic data be accurately allied with other genome readouts, heralding 'integromics'-driven personalized medicine for CCA patients.

**FIGURE TEXT**

**Figure 1. Genomic alterations of epigenetic regulators in CCA patients.** Plot depicting the frequency of mutation and structural alterations to epigenetic regulators in TCGA biliary tract cancer tissue samples. Data were assessed from whole-exome sequencing experiments conducted by TCGA CHOL consortium. Categories of epigenetic enzymes were mined from EpiFactors database (http://epifactors.autosome.ru/).

**Figure 2. Schematic representation of pathways modulated by long non-coding RNAs in CCA cells.** H19, HULC and MALAT1 are upregulated in CCA cells. LncRNA-miR sponging has been shown for these lncRNAs and miR-let-7a/let-7b, miR-372/miR-373 and miR-204, respectively. In this process, sponging has been demonstrated to increase the expression of inflammation–related genes, IL-6 and CXCR4, causing abnormal inflammatory state, which is involved in the pre-neoplastic onset and progression of the disease. Secondly, lncRNA (NEAT1) is upregulated in CCA cells when the level of its upstream regulator BAP1 is attenuated, thereby, enhancing sensitivity to gemcitabine treatment. Third, a high expression level of lncRNA AFAP1-AS1 promotes growth and metastasis of CCA cells. Also, increased expression of the mitochondrial enzyme encoded by the gene *CPS1* and its lncRNA (CPS1-IT1) have been shown to promote CCA proliferation. These data emphasize the potential of lncRNAs as diagnostic and prognostic markers as well as putative regulators of drug response in CCA.

**Figure 3. DNA methylome dysregulation during cholangiocyte transformation.** The malignant transformation of normal cholangiocytes is accompanied by gross insult to the DNA methylome. This is mediated through diverse mechanisms which impede DNA methylation catalysis, including mutations (e.g. IDH1, IDH2), epigenetic alterations (e.g. DNMT-targeting miRs) and autocrine factors (e.g. elevated IL-6

levels). The CCA methylome displays several hallmarks characteristic of cancer. These include (1) promoter DNA hypermethylation of genes with tumor suppressive functions, resulting in gene body hypomethylation and transcriptional silencing; (2) promoter hypomethylation of genes with oncogenic functions, leading to gene body hypermethylation and transcriptional activation; and (3) loss of methylation at intergenic regions, promoting reactivation of endoparasitic sequences and genomic instability. *5hmC: 5-hydroxymethylcytosine. DNMT: DNA Methyltransferase. TSG: Tumor Suppressor Gene.*

**Figure 4. SWI/SNF remodeling complex dysfunction in cholangiocarcinogenesis.** Cholangiocarcinogenesis is a multi-step process involving the degeneration of normal bile ducts to hyper-proliferative pre-malignant lesions *in situ* (e.g. BiIN, IPN), followed by acquisition of an invasive phenotype to form primary adenocarcinoma. SWI/SNF is a multi-protein chromatin remodeling complex responsible for directed repositioning of nucleosomes, enabling efficient DNA repair, transcriptional homeostasis and genome stability. Recurrent mutations in SWI/SNF complex members, *ARID1A* and *PBRM1*, have been detected in CCA patients and have been specifically identified to occur after the development of carcinoma *in situ*. The broad consequences of such late mutational events (likely DNA misrepair/damage, transcriptome dysregulation and genomic instability) remain to be characterized in CCA and, given their timing during disease pathogenesis, SWI/SNF dysfunction may contribute to manifestation of the invasive phenotype. *BiIN: Biliary Intraepithelial Neoplasia. IPN: Intra-ductal Papillary Neoplasia. SWI/SNF: SWItch/Sucrose Non-Fermentable.*

**Figure 5: A rational framework for conducting EWAS in CCA.** Expansion of epigenetic analyses from locus-specific to (epi)genome-wide scales presents new challenges for experimental design. These include careful study population stratification to avoid potential confounding factors (age, gender, hepatitis infection, alcohol exposure, NAFLD/NASH), cellular deconvolution of epigenetic signals (using reference-based or reference-free statistical algorithms, microdissection) and cautious inference of causation or indirect causation (through matched 'omic' controls and functional editing of epimutations). Prospective consideration of such limitations is warranted in CCA to maximize likelihood for successful translation of findings into clinical trials. *EWAS: Epigenome-Wide Association Study. NAFLD: Non-Alcoholic Fatty Liver Disease. NASH: Non-Alcoholic Steatohepatitis.*

**REFERENCES:**


[1] K.J. Yao, S. Jabbour, N. Parekh, Y. Lin, R.A. Moss, Increasing mortality in the United States from cholangiocarcinoma: an analysis of the National Center for Health Statistics Database, BMC Gastroenterol, 16 (2016) 117.

[2] J.M. Llovet, A. Villanueva, A. Lachenmayer, R.S. Finn, Advances in targeted therapies for hepatocellular carcinoma in the genomic era, Nat Rev Clin Oncol, 12 (2015) 436.

[3] J.M. Banales, V. Cardinale, G. Carpino, M. Marzioni, J.B. Andersen, P. Invernizzi, G.E. Lind, T. Folseraas, S.J. Forbes, L. Fouassier, A. Geier, D.F. Calvisi, J.C. Mertens, M. Trauner, A. Benedetti, L. Maroni, J. Vaquero, R.I. Macias, C. Raggi, M.J. Perugorria, E. Gaudio, K.M. Boberg, J.J. Marin, D. Alvaro, Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA), Nat Rev Gastroenterol Hepatol, 13 (2016) 261-280.

[4] F. Farshidfar, S. Zheng, M.C. Gingras, Y. Newton, J. Shih, A.G. Robertson, T. Hinoue, K.A. Hoadley, E.A. Gibb, J. Roszik, K.R. Covington, C.C. Wu, E. Shinbrot, N. Stransky, A. Hegde, J.D. Yang, E. Reznik, S. Sadeghi, C.S. Pedamallu, A.I. Ojesina, J.M. Hess, J.T. Auman, S.K. Rhie, R. Bowlby, M.J. Borad, N. Cancer Genome Atlas, A.X. Zhu, J.M. Stuart, C. Sander, R. Akbani, A.D. Cherniack, V. Deshpande, T. Mounajjed, W.C. Foo, M.S. Torbenson, D.E. Kleiner, P.W. Laird, D.A. Wheeler, A.J. McRee, O.F. Bathe, J.B. Andersen, N. Bardeesy, L.R. Roberts, L.N. Kwong, Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles, Cell Rep, 18 (2017) 2780-2794.

[5] A. Fujimoto, M. Furuta, Y. Shiraishi, K. Gotoh, Y. Kawakami, K. Arihiro, T. Nakamura, M. Ueno, S. Ariizumi, H.H. Nguyen, D. Shigemizu, T. Abe, K.A. Boroevich, K. Nakano, A. Sasaki, R. Kitada, K. Maejima, Y. Yamamoto, H. Tanaka, T. Shibuya, T. Shibata, H. Ojima, K. Shimada, S. Hayami, Y. Shigekawa, H. Aikata, H. Ohdan, S. Marubashi, T. Yamada, M. Kubo, S. Hirano, O. Ishikawa, M. Yamamoto, H. Yamaue, K. Chayama, S. Miyano, T. Tsunoda, H. Nakagawa, Whole-genome mutational landscape of liver cancers displaying biliary phenotype reveals hepatitis impact and molecular diversity, Nat Commun, 6 (2015) 6120.

[6] Y. Jiao, T.M. Pawlik, R.A. Anders, F.M. Selaru, M.M. Streppel, D.J. Lucas, N. Niknafs, V.B. Guthrie, A. Maitra, P. Argani, G.J. Offerhaus, J.C. Roa, L.R. Roberts, G.J. Gores, I. Popescu, S.T. Alexandrescu, S. Dima, M. Fassan, M. Simbolo, A. Mafficini, P. Capelli, R.T. Lawlor, A. Ruzzenente, A. Guglielmi, G. Tortora, F. de Braud, A. Scarpa, W. Jarnagin, D. Klimstra, R. Karchin, V.E. Velculescu, R.H. Hruban, B. Vogelstein, K.W. Kinzler, N. Papadopoulos, L.D. Wood, Exome sequencing identifies frequent inactivating mutations in BAP1, ARID1A and PBRM1 in intrahepatic cholangiocarcinomas, Nat Genet, 45 (2013) 1470-1473.

[7] J.B. Andersen, B. Spee, B.R. Blechacz, I. Avital, M. Komuta, A. Barbour, E.A. Conner, M.C. Gillen, T. Roskams, L.R. Roberts, V.M. Factor, S.S. Thorgeirsson, Genomic and genetic characterization of cholangiocarcinoma identifies therapeutic targets for tyrosine kinase inhibitors, Gastroenterology, 142 (2012) 1021-1031 e1015.

[8] D. Sia, Y. Hoshida, A. Villanueva, S. Roayaie, J. Ferrer, B. Tabak, J. Peix, M. Sole, V. Tovar, C. Alsinet, H. Cornella, B. Klotzle, J.B. Fan, C. Cotsoglou, S.N. Thung, J. Fuster, S. Waxman, J.C. Garcia-Valdecasas, J. Bruix, M.E. Schwartz, R. Beroukhim, V. Mazzaferro, J.M. Llovet, Integrative molecular analysis of intrahepatic cholangiocarcinoma reveals 2 classes that have different outcomes, Gastroenterology, 144 (2013) 829-840.

[9] M.P. Hitchins, Constitutional epimutation as a mechanism for cancer causality and heritability?, Nat Rev Cancer, 15 (2015) 625-634.

[10] R. Sriraksa, C. Zeller, W. Dai, A. Siddiq, A.J. Walley, T. Limpaiboon, R. Brown, Aberrant DNA methylation at genes associated with a stem cell-like phenotype in cholangiocarcinoma tumors, Cancer Prev Res (Phila), 6 (2013) 1348-1355.

[11] B. Goeppert, C. Konermann, C.R. Schmidt, O. Bogatyrova, L. Geiselhart, C. Ernst, L. Gu, N. Becker, M. Zucknick, A. Mehrabi, M. Hafezi, F. Klauschen, A. Stenzinger, A. Warth, K. Breuhahn, M. Renner, W. Weichert, P. Schirmacher, C. Plass, D. Weichenhan, Global alterations of DNA methylation in cholangiocarcinoma target the Wnt signaling pathway, Hepatology, 59 (2014) 544-554.

[12] A. Esparza-Baquer, I. Labiano, L. Bujanda, M.J. Perugorria, J.M. Banales, MicroRNAs in cholangiopathies: Potential diagnostic and therapeutic tools, Clin Res Hepatol Gastroenterol, 40 (2016) 15-27.
[13] R.C. Lee, R.L. Feinbaum, V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell, 75 (1993) 843-854.
[14] L.P. Lim, M.E. Glasner, S. Yekta, C.B. Burge, D.P. Bartel, Vertebrate microRNA genes, Science, 299 (2003) 1540.
[15] A. Budhu, J. Ji, X.W. Wang, The clinical potential of microRNAs, Journal of hematology & oncology, 3 (2010) 37.
[16] F.M. Selaru, A.V. Olaru, T. Kan, S. David, Y. Cheng, Y. Mori, J. Yang, B. Paun, Z. Jin, R. Agarwal, J.P. Hamilton, J. Abraham, C. Georgiades, H. Alvarez, P. Vivekanandan, W. Yu, A. Maitra, M. Torbenson, P.J. Thuluvath, G.J. Gores, N.F. LaRusso, R. Hruban, S.J. Meltzer, MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3, Hepatology, 49 (2009) 1595-1601.
[17] Q. Huang, L. Liu, C.H. Liu, H. You, F. Shao, F. Xie, X.S. Lin, S.Y. Hu, C.H. Zhang, MicroRNA-21 regulates the invasion and metastasis in cholangiocarcinoma and may be a potential biomarker for cancer prognosis, Asian Pac J Cancer Prev, 14 (2013) 829-834.
[18] F. Meng, R. Henson, M. Lang, H. Wehbe, S. Maheshwari, J.T. Mendell, J. Jiang, T.D. Schmittgen, T. Patel, Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines, Gastroenterology, 130 (2006) 2113-2129.
[19] C. Correa-Gallego, D. Maddalo, A. Doussot, N. Kemeny, T.P. Kingham, P.J. Allen, M.I. D'Angelica, R.P. DeMatteo, D. Betel, D. Klimstra, W.R. Jarnagin, A. Ventura, Circulating Plasma Levels of MicroRNA-21 and MicroRNA-221 Are Potential Diagnostic Markers for Primary Intrahepatic Cholangiocarcinoma, PLoS One, 11 (2016) e0163699.
[20] N. Namwat, P. Chusorn, W. Loilome, A. Techasen, J. Puetkasichonpasutha, C. Pairojkul, N. Khuntikeo, P. Yongvanit, Expression profiles of oncomir miR-21 and tumor suppressor let-7a in the progression of opisthorchiasis-associated cholangiocarcinoma, Asian Pac J Cancer Prev, 13 Suppl (2012) 65-69.
[21] R. Urbas, C. Mayr, E. Klieser, J. Fuereder, D. Bach, S. Stattner, F. Primavesi, T. Jaeger, S. Stanzer, A.L. Ress, M. Loffelberger, A. Wagner, F. Berr, M. Ritter, M. Pichler, D. Neureiter, T. Kiesslich, Relevance of MicroRNA200 Family and MicroRNA205 for Epithelial to Mesenchymal Transition and Clinical Outcome in Biliary Tract Cancer Patients, Int J Mol Sci, 17 (2016).
[22] N. Oishi, M.R. Kumar, S. Roessler, J. Ji, M. Forgues, A. Budhu, X. Zhao, J.B. Andersen, Q.H. Ye, H.L. Jia, L.X. Qin, T. Yamashita, H.G. Woo, Y.J. Kim, S. Kaneko, Z.Y. Tang, S.S. Thorgeirsson, X.W. Wang, Transcriptomic profiling reveals hepatic stem-like gene signatures and interplay of miR-200c and epithelial-mesenchymal transition in intrahepatic cholangiocarcinoma, Hepatology, 56 (2012) 1792-1803.
[23] F. Peng, J. Jiang, Y. Yu, R. Tian, X. Guo, X. Li, M. Shen, M. Xu, F. Zhu, C. Shi, J. Hu, M. Wang, R. Qin, Direct targeting of SUZ12/ROCK2 by miR-200b/c inhibits cholangiocarcinoma tumourigenesis and metastasis, Br J Cancer, 109 (2013) 3092-3104.
[24] P. Qiao, G. Li, W. Bi, L. Yang, L. Yao, D. Wu, microRNA-34a inhibits epithelial mesenchymal transition in human cholangiocarcinoma by targeting Smad4 through transforming growth factor-beta/Smad pathway, BMC Cancer, 15 (2015) 469.
[25] Y.H. Qiu, Y.P. Wei, N.J. Shen, Z.C. Wang, T. Kan, W.L. Yu, B. Yi, Y.J. Zhang, miR-204 inhibits epithelial to mesenchymal transition by targeting slug in intrahepatic cholangiocarcinoma cells, Cell Physiol Biochem, 32 (2013) 1331-1341.
[26] L. Chen, H.X. Yan, W. Yang, L. Hu, L.X. Yu, Q. Liu, L. Li, D.D. Huang, J. Ding, F. Shen, W.P. Zhou, M.C. Wu, H.Y. Wang, The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma, J Hepatol, 50 (2009) 358-369.
[27] B. Li, Q. Han, Y. Zhu, Y. Yu, J. Wang, X. Jiang, Down-regulation of miR-214 contributes to intrahepatic cholangiocarcinoma metastasis by targeting Twist, FEBS J, 279 (2012) 2393-2398.

[28] J. Li, L. Yao, G. Li, D. Ma, C. Sun, S. Gao, P. Zhang, F. Gao, miR-221 Promotes Epithelial-Mesenchymal Transition through Targeting PTEN and Forms a Positive Feedback Loop with beta-catenin/c-Jun Signaling Pathway in Extra-Hepatic Cholangiocarcinoma, PLoS One, 10 (2015) e0141168.
[29] F. Meng, H. Wehbe-Janek, R. Henson, H. Smith, T. Patel, Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes, Oncogene, 27 (2008) 378-386.
[30] C. Braconi, N. Huang, T. Patel, MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes, Hepatology, 51 (2010) 881-890.
[31] Y. Chen, J. Luo, R. Tian, H. Sun, S. Zou, miR-373 negatively regulates methyl-CpG-binding domain protein 2 (MBD2) in hilar cholangiocarcinoma, Dig Dis Sci, 56 (2011) 1693-1701.
[32] Y.J. Chen, J. Luo, G.Y. Yang, K. Yang, S.Q. Wen, S.Q. Zou, Mutual regulation between microRNA-373 and methyl-CpG-binding domain protein 2 in hilar cholangiocarcinoma, World J Gastroenterol, 18 (2012) 3849-3861.
[33] B. Goeppert, C. Ernst, C. Baer, S. Roessler, M. Renner, A. Mehrabi, M. Hafezi, A. Pathil, A. Warth, A. Stenzinger, W. Weichert, M. Bahr, R. Will, P. Schirmacher, C. Plass, D. Weichenhan, Cadherin-6 is a putative tumor suppressor and target of epigenetically dysregulated miR-429 in cholangiocarcinoma, Epigenetics, 11 (2016) 780-790.
[34] T. Palumbo, G.A. Poultsides, G. Kouraklis, T. Liakakos, A. Drakaki, G. Peros, M. Hatziapostolou, D. Iliopoulos, A functional microRNA library screen reveals miR-410 as a novel anti-apoptotic regulator of cholangiocarcinoma, BMC Cancer, 16 (2016) 353.
[35] K.Y. Lin, H. Ye, B.W. Han, W.T. Wang, P.P. Wei, B. He, X.J. Li, Y.Q. Chen, Genome-wide screen identified let-7c/miR-99a/miR-125b regulating tumor progression and stem-like properties in cholangiocarcinoma, Oncogene, 35 (2016) 3376-3386.
[36] H. Isomoto, S. Kobayashi, N.W. Werneburg, S.F. Bronk, M.E. Guicciardi, D.A. Frank, G.J. Gores, Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells, Hepatology, 42 (2005) 1329-1338.
[37] W.T. Wang, H. Ye, P.P. Wei, B.W. Han, B. He, Z.H. Chen, Y.Q. Chen, LncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner, J Hematol Oncol, 9 (2016) 117.
[38] F. Meng, R. Henson, H. Wehbe-Janek, H. Smith, Y. Ueno, T. Patel, The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes, J Biol Chem, 282 (2007) 8256-8264.
[39] X. Tan, Z. Huang, X. Li, Long Non-Coding RNA MALAT1 Interacted with miR-204 to Modulates Human Hilar Cholangiocarcinoma Proliferation, Migration and Invasion by Targeting CXCR4, J Cell Biochem, (2017).
[40] M. Parasramka, I.K. Yan, X. Wang, P. Nguyen, A. Matsuda, S. Maji, C. Foye, Y. Asmann, T. Patel, BAP1 dependent expression of long non-coding RNA NEAT-1 contributes to sensitivity to gemcitabine in cholangiocarcinoma, Mol Cancer, 16 (2017) 22.
[41] S.L. Ma, A.J. Li, Z.Y. Hu, F.S. Shang, M.C. Wu, Coexpression of the carbamoylphosphate synthase 1 gene and its long noncoding RNA correlates with poor prognosis of patients with intrahepatic cholangiocarcinoma, Mol Med Rep, 12 (2015) 7915-7926.
[42] X. Shi, H. Zhang, M. Wang, X. Xu, Y. Zhao, R. He, M. Zhang, M. Zhou, X. Li, F. Peng, C. Shi, M. Shen, X. Wang, X. Guo, R. Qin, LncRNA AFAP1-AS1 promotes growth and metastasis of cholangiocarcinoma cells, Oncotarget, (2017).
[43] P. Johnsson, A. Ackley, L. Vidarsdottir, W.O. Lui, M. Corcoran, D. Grander, K.V. Morris, A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells, Nat Struct Mol Biol, 20 (2013) 440-446.
[44] J. Wang, H. Xie, Q. Ling, D. Lu, Z. Lv, R. Zhuang, Z. Liu, X. Wei, L. Zhou, X. Xu, S. Zheng, Coding-noncoding gene expression in intrahepatic cholangiocarcinoma, Transl Res, 168 (2016) 107-121.

[45] L. Lv, M. Wei, P. Lin, Z. Chen, P. Gong, Z. Quan, Z. Tang, Integrated mRNA and lncRNA expression profiling for exploring metastatic biomarkers of human intrahepatic cholangiocarcinoma, Am J Cancer Res, 7 (2017) 688-699.
[46] A.P. Feinberg, R. Ohlsson, S. Henikoff, The epigenetic progenitor origin of human cancer, Nat Rev Genet, 7 (2006) 21-33.
[47] S.B. Baylin, P.A. Jones, Epigenetic Determinants of Cancer, Cold Spring Harb Perspect Biol, 8 (2016).
[48] S. Udali, P. Guarini, S. Moruzzi, A. Ruzzenente, S.A. Tammen, A. Guglielmi, S. Conci, P. Pattini, O. Olivieri, R. Corrocher, S.W. Choi, S. Friso, Global DNA methylation and hydroxymethylation differ in hepatocellular carcinoma and cholangiocarcinoma and relate to survival rate, Hepatology, 62 (2015) 496-504.
[49] Z.R. Dong, C. Zhang, J.B. Cai, P.F. Zhang, G.M. Shi, D.M. Gao, H.C. Sun, S.J. Qiu, J. Zhou, A.W. Ke, J. Fan, Role of 5-hydroxymethylcytosine level in diagnosis and prognosis prediction of intrahepatic cholangiocarcinoma, Tumour Biol, 36 (2015) 2763-2771.
[50] Y. Gao, M. Yang, Z. Jiang, B.A. Woda, A.M. Mercurio, J. Qin, X. Huang, F. Zhang, IMP3 expression is associated with poor outcome and epigenetic deregulation in intrahepatic cholangiocarcinoma, Hum Pathol, 45 (2014) 1184-1191.
[51] H.M. Vedeld, K. Andresen, I.A. Eilertsen, A. Nesbakken, R. Seruca, I.P. Gladhaug, E. Thiis-Evensen, T.O. Rognum, K.M. Boberg, G.E. Lind, The novel colorectal cancer biomarkers CDO1, ZSCAN18 and ZNF331 are frequently methylated across gastrointestinal cancers, Int J Cancer, 136 (2015) 844-853.
[52] L. Xiaofang, T. Kun, Y. Shaoping, W. Zaiqiu, S. Hailong, Correlation between promoter methylation of p14(ARF), TMS1/ASC, and DAPK, and p53 mutation with prognosis in cholangiocarcinoma, World J Surg Oncol, 10 (2012) 5.
[53] S. Dachrut, S. Banthaisong, M. Sripa, A. Paeyao, C. Ho, S.A. Lee, C. Kosinski, M.A. Patil, J. Zhang, X. Chen, B. Sripa, C. Pairojkul, DNA copy-number loss on 1p36.1 harboring RUNX3 with promoter hypermethylation and associated loss of RUNX3 expression in liver fluke-associated intrahepatic cholangiocarcinoma, Asian Pac J Cancer Prev, 10 (2009) 575-582.
[54] Y. Shu, B. Wang, J. Wang, J.M. Wang, S.Q. Zou, Identification of methylation profile of HOX genes in extrahepatic cholangiocarcinoma, World J Gastroenterol, 17 (2011) 3407-3419.
[55] M. Merino-Azpitarte, E. Lozano, M.J. Perugorria, A. Esparza-Baquer, O. Erice, A. Santos-Laso, C.J. O'Rourke, J.B. Andersen, R. Jimenez-Aguero, A. Lacasta, M. D'Amato, O. Briz, N. Jalan-Sakrikar, R.C. Huebert, K.M. Thelen, S.A. Gradilone, A.M. Aransay, J.L. Lavin, M.G. Fernandez-Barrena, A. Matheu, M. Marzioni, G.J. Gores, L. Bujanda, J.J. Marin, J.M. Banales, SOX17 regulates cholangiocyte differentiation and acts as a tumor suppressor in cholangiocarcinoma, J Hepatol, (2017).
[56] J.U. Marquardt, J.B. Andersen, S.S. Thorgeirsson, Functional and genetic deconstruction of the cellular origin in liver cancer, Nat Rev Cancer, 15 (2015) 653-667.
[57] B. Klump, C.J. Hsieh, S. Dette, K. Holzmann, R. Kiebetalich, M. Jung, U. Sinn, M. Ortner, R. Porschen, M. Gregor, Promoter methylation of INK4a/ARF as detected in bile-significance for the differential diagnosis in biliary disease, Clin Cancer Res, 9 (2003) 1773-1778.
[58] A. Ishikawa, M. Sasaki, Y. Sato, S. Ohira, M.F. Chen, S.F. Huang, K. Oda, Y. Nimura, Y. Nakanuma, Frequent p16ink4a inactivation is an early and frequent event of intraductal papillary neoplasm of the liver arising in hepatolithiasis, Hum Pathol, 35 (2004) 1505-1514.
[59] B. Zeng, Z. Li, R. Chen, N. Guo, J. Zhou, Q. Zhou, Q. Lin, D. Cheng, Q. Liao, L. Zheng, Y. Gong, Epigenetic regulation of miR-124 by hepatitis C virus core protein promotes migration and invasion of intrahepatic cholangiocarcinoma cells by targeting SMYD3, FEBS Lett, 586 (2012) 3271-3278.
[60] B.H. Kim, N.Y. Cho, S.H. Shin, H.J. Kwon, J.J. Jang, G.H. Kang, CpG island hypermethylation and repetitive DNA hypomethylation in premalignant lesion of extrahepatic cholangiocarcinoma, Virchows Arch, 455 (2009) 343-351.
[61] K.O. Uhm, J.O. Lee, Y.M. Lee, E.S. Lee, H.S. Kim, S.H. Park, Aberrant DNA methylation of integrin alpha4: a potential novel role for metastasis of cholangiocarcinoma, J Cancer Res Clin Oncol, 136 (2010) 187-194.

[62] P. Wang, Q. Dong, C. Zhang, P.F. Kuan, Y. Liu, W.R. Jeck, J.B. Andersen, W. Jiang, G.L. Savich, T.X. Tan, J.T. Auman, J.M. Hoskins, A.D. Misher, C.D. Moser, S.M. Yourstone, J.W. Kim, K. Cibulskis, G. Getz, H.V. Hunt, S.S. Thorgeirsson, L.R. Roberts, D. Ye, K.L. Guan, Y. Xiong, L.X. Qin, D.Y. Chiang, Mutations in isocitrate dehydrogenase 1 and 2 occur frequently in intrahepatic cholangiocarcinomas and share hypermethylation targets with glioblastomas, Oncogene, 32 (2013) 3091-3100.
[63] J.A. Losman, W.G. Kaelin, Jr., What a difference a hydroxyl makes: mutant IDH, (R)-2-hydroxyglutarate, and cancer, Genes Dev, 27 (2013) 836-852.
[64] H. Wehbe, R. Henson, F. Meng, J. Mize-Berge, T. Patel, Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression, Cancer Res, 66 (2006) 10517-10524.
[65] H. Isomoto, J.L. Mott, S. Kobayashi, N.W. Werneburg, S.F. Bronk, S. Haan, G.J. Gores, Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing, Gastroenterology, 132 (2007) 384-396.
[66] W. Cheng, Y. Qi, L. Tian, B. Wang, W. Huang, Y. Chen, Dicer promotes tumorigenesis by translocating to nucleus to promote SFRP1 promoter methylation in cholangiocarcinoma cells, Cell Death Dis, 8 (2017) e2628.
[67] K. Andresen, K.M. Boberg, H.M. Vedeld, H. Honne, M. Hektoen, C.A. Wadsworth, O.P. Clausen, T.H. Karlsen, A. Foss, O. Mathisen, E. Schrumpf, R.A. Lothe, G.E. Lind, Novel target genes and a valid biomarker panel identified for cholangiocarcinoma, Epigenetics, 7 (2012) 1249-1257.
[68] V. Branchi, P. Schaefer, A. Semaan, A. Kania, P. Lingohr, J.C. Kalff, N. Schafer, G. Kristiansen, D. Dietrich, H. Matthaei, Promoter hypermethylation of SHOX2 and SEPT9 is a potential biomarker for minimally invasive diagnosis in adenocarcinomas of the biliary tract, Clin Epigenetics, 8 (2016) 133.
[69] K. Andresen, K.M. Boberg, H.M. Vedeld, H. Honne, P. Jebsen, M. Hektoen, C.A. Wadsworth, O.P. Clausen, K.E. Lundin, V. Paulsen, A. Foss, O. Mathisen, L. Aabakken, E. Schrumpf, R.A. Lothe, G.E. Lind, Four DNA methylation biomarkers in biliary brush samples accurately identify the presence of cholangiocarcinoma, Hepatology, 61 (2015) 1651-1659.
[70] A. Villanueva, A. Portela, S. Sayols, C. Battiston, Y. Hoshida, J. Mendez-Gonzalez, S. Imbeaud, E. Letouze, V. Hernandez-Gea, H. Cornella, R. Pinyol, M. Sole, J. Fuster, J. Zucman-Rossi, V. Mazzaferro, M. Esteller, J.M. Llovet, H. Consortium, DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma, Hepatology, 61 (2015) 1945-1956.
[71] K. Nakamura, K. Nakabayashi, K. Htet Aung, K. Aizawa, N. Hori, J. Yamauchi, K. Hata, A. Tanoue, DNA methyltransferase inhibitor zebularine induces human cholangiocarcinoma cell death through alteration of DNA methylation status, PLoS One, 10 (2015) e0120545.
[72] J.B. Andersen, V.M. Factor, J.U. Marquardt, C. Raggi, Y.H. Lee, D. Seo, E.A. Conner, S.S. Thorgeirsson, An integrated genomic and epigenomic approach predicts therapeutic response to zebularine in human liver cancer, Sci Transl Med, 2 (2010) 54ra77.
[73] Y. Morine, M. Shimada, S. Iwahashi, T. Utsunomiya, S. Imura, T. Ikemoto, H. Mori, J. Hanaoka, H. Miyake, Role of histone deacetylase expression in intrahepatic cholangiocarcinoma, Surgery, 151 (2012) 412-419.
[74] J.C. He, W. Yao, J.M. Wang, P. Schemmer, Y. Yang, Y. Liu, Y.W. Qian, W.P. Qi, J. Zhang, Q. Shen, T. Yang, TACC3 overexpression in cholangiocarcinoma correlates with poor prognosis and is a potential anti-cancer molecular drug target for HDAC inhibitors, Oncotarget, 7 (2016) 75441-75456.
[75] S.A. Gradilone, B.N. Radtke, P.S. Bogert, B.Q. Huang, G.B. Gajdos, N.F. LaRusso, HDAC6 inhibition restores ciliary expression and decreases tumor growth, Cancer Res, 73 (2013) 2259-2270.
[76] Y. Mizuguchi, S. Specht, J.G. Lunz, 3rd, K. Isse, N. Corbitt, T. Takizawa, A.J. Demetris, SPRR2A enhances p53 deacetylation through HDAC1 and down regulates p21 promoter activity, BMC Mol Biol, 13 (2012) 20.
[77] H. Wang, C. Li, Z. Jian, Y. Ou, J. Ou, TGF-beta1 Reduces miR-29a Expression to Promote Tumorigenicity and Metastasis of Cholangiocarcinoma by Targeting HDAC4, PLoS One, 10 (2015) e0136703.

[78] V. Baradari, M. Hopfner, A. Huether, D. Schuppan, H. Scherubl, Histone deacetylase inhibitor MS-275 alone or combined with bortezomib or sorafenib exhibits strong antiproliferative action in human cholangiocarcinoma cells, World J Gastroenterol, 13 (2007) 4458-4466.
[79] R. Sriraksa, T. Limpaiboon, Histone deacetylases and their inhibitors as potential therapeutic drugs for cholangiocarcinoma - cell line findings, Asian Pac J Cancer Prev, 14 (2013) 2503-2508.
[80] B. Wang, R. Yang, Y. Wu, H. Li, Z. Hu, Y. Chen, S. Zou, Sodium valproate inhibits the growth of human cholangiocarcinoma in vitro and in vivo, Gastroenterol Res Pract, 2013 (2013) 374593.
[81] S. Iwahashi, T. Utsunomiya, S. Imura, Y. Morine, T. Ikemoto, Y. Arakawa, Y. Saito, D. Ishikawa, M. Shimada, Effects of valproic acid in combination with S-1 on advanced pancreatobiliary tract cancers: clinical study phases I/II, Anticancer Res, 34 (2014) 5187-5191.
[82] T.W. Kwak, D.H. Kim, Y.I. Jeong, D.H. Kang, Antitumor activity of vorinostat-incorporated nanoparticles against human cholangiocarcinoma cells, J Nanobiotechnology, 13 (2015) 60.
[83] S. Nakagawa, H. Okabe, Y. Sakamoto, H. Hayashi, D. Hashimoto, N. Yokoyama, K. Sakamoto, H. Kuroki, K. Mima, H. Nitta, K. Imai, A. Chikamoto, M. Watanabe, T. Beppu, H. Baba, Enhancer of zeste homolog 2 (EZH2) promotes progression of cholangiocarcinoma cells by regulating cell cycle and apoptosis, Ann Surg Oncol, 20 Suppl 3 (2013) S667-675.
[84] S. Nakagawa, Y. Sakamoto, H. Okabe, H. Hayashi, D. Hashimoto, N. Yokoyama, R. Tokunaga, K. Sakamoto, H. Kuroki, K. Mima, T. Beppu, H. Baba, Epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A inhibits the growth of cholangiocarcinoma cells, Oncol Rep, 31 (2014) 983-988.
[85] B. Tang, J. Du, Y. Li, F. Tang, Z. Wang, S. He, EZH2 elevates the proliferation of human cholangiocarcinoma cells through the downregulation of RUNX3, Med Oncol, 31 (2014) 271.
[86] W. Chan-On, M.L. Nairismagi, C.K. Ong, W.K. Lim, S. Dima, C. Pairojkul, K.H. Lim, J.R. McPherson, I. Cutcutache, H.L. Heng, L. Ooi, A. Chung, P. Chow, P.C. Cheow, S.Y. Lee, S.P. Choo, I.B. Tan, D. Duda, A. Nastase, S.S. Myint, B.H. Wong, A. Gan, V. Rajasegaran, C.C. Ng, S. Nagarajan, A. Jusakul, S. Zhang, P. Vohra, W. Yu, D. Huang, P. Sithithaworn, P. Yongvanit, S. Wongkham, N. Khuntikeo, V. Bhudhisawasdi, I. Popescu, S.G. Rozen, P. Tan, B.T. Teh, Exome sequencing identifies distinct mutational patterns in liver fluke-related and non-infection-related bile duct cancers, Nat Genet, 45 (2013) 1474-1478.
[87] S. Zou, J. Li, H. Zhou, C. Frech, X. Jiang, J.S. Chu, X. Zhao, Y. Li, Q. Li, H. Wang, J. Hu, G. Kong, M. Wu, C. Ding, N. Chen, H. Hu, Mutational landscape of intrahepatic cholangiocarcinoma, Nat Commun, 5 (2014) 5696.
[88] H. Nakamura, Y. Arai, Y. Totoki, T. Shirota, A. Elzawahry, M. Kato, N. Hama, F. Hosoda, T. Urushidate, S. Ohashi, N. Hiraoka, H. Ojima, K. Shimada, T. Okusaka, T. Kosuge, S. Miyagawa, T. Shibata, Genomic spectra of biliary tract cancer, Nat Genet, 47 (2015) 1003-1010.
[89] J. Weber, R. Ollinger, M. Friedrich, U. Ehmer, M. Barenboim, K. Steiger, I. Heid, S. Mueller, R. Maresch, T. Engleitner, N. Gross, U. Geumann, B. Fu, A. Segler, D. Yuan, S. Lange, A. Strong, J. de la Rosa, I. Esposito, P. Liu, J. Cadinanos, G.S. Vassiliou, R.M. Schmid, G. Schneider, K. Unger, F. Yang, R. Braren, M. Heikenwalder, I. Varela, D. Saur, A. Bradley, R. Rad, CRISPR/Cas9 somatic multiplex-mutagenesis for high-throughput functional cancer genomics in mice, Proc Natl Acad Sci U S A, 112 (2015) 13982-13987.
[90] M. Sasaki, T. Nitta, Y. Sato, Y. Nakanuma, Loss of ARID1A Expression Presents a Novel Pathway of Carcinogenesis in Biliary Carcinomas, Am J Clin Pathol, 145 (2016) 815-825.
[91] C. Luchini, S.A. Robertson, S.M. Hong, M. Felsenstein, R.A. Anders, A. Pea, A. Nottegar, N. Veronese, J. He, M.J. Weiss, P. Capelli, A. Scarpa, P. Argani, P. Kapur, L.D. Wood, PBRM1 loss is a late event during the development of cholangiocarcinoma, Histopathology, (2017).
[92] K.B. Michels, A.M. Binder, S. Dedeurwaerder, C.B. Epstein, J.M. Greally, I. Gut, E.A. Houseman, B. Izzi, K.T. Kelsey, A. Meissner, A. Milosavljevic, K.D. Siegmund, C. Bock, R.A. Irizarry, Recommendations for the design and analysis of epigenome-wide association studies, Nat Methods, 10 (2013) 949-955.
[93] E. Birney, G.D. Smith, J.M. Greally, Epigenome-wide Association Studies and the Interpretation of Disease -Omics, PLoS Genet, 12 (2016) e1006105.

[94] R.A. Hlady, R.L. Tiedemann, W. Puszyk, I. Zendejas, L.R. Roberts, J.H. Choi, C. Liu, K.D. Robertson, Epigenetic signatures of alcohol abuse and hepatitis infection during human hepatocarcinogenesis, Oncotarget, 5 (2014) 9425-9443.
[95] E.A. Houseman, M.L. Kile, D.C. Christiani, T.A. Ince, K.T. Kelsey, C.J. Marsit, Reference-free deconvolution of DNA methylation data and mediation by cell composition effects, BMC Bioinformatics, 17 (2016) 259.
[96] X.S. Liu, H. Wu, X. Ji, Y. Stelzer, X. Wu, S. Czauderna, J. Shu, D. Dadon, R.A. Young, R. Jaenisch, Editing DNA Methylation in the Mammalian Genome, Cell, 167 (2016) 233-247 e217.
[97] I.B. Hilton, A.M. D'Ippolito, C.M. Vockley, P.I. Thakore, G.E. Crawford, T.E. Reddy, C.A. Gersbach, Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers, Nat Biotechnol, 33 (2015) 510-517.
[98] S.J. Liu, M.A. Horlbeck, S.W. Cho, H.S. Birk, M. Malatesta, D. He, F.J. Attenello, J.E. Villalta, M.Y. Cho, Y. Chen, M.A. Mandegar, M.P. Olvera, L.A. Gilbert, B.R. Conklin, H.Y. Chang, J.S. Weissman, D.A. Lim, CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells, Science, 355 (2017).
[99] L.J. Wang, C.C. He, X. Sui, M.J. Cai, C.Y. Zhou, J.L. Ma, L. Wu, H. Wang, S.X. Han, Q. Zhu, MiR-21 promotes intrahepatic cholangiocarcinoma proliferation and growth in vitro and in vivo by targeting PTPN14 and PTEN, Oncotarget, 6 (2015) 5932-5946.
[100] X.Y. Zhong, J.H. Yu, W.G. Zhang, Z.D. Wang, Q. Dong, S. Tai, Y.F. Cui, H. Li, MicroRNA-421 functions as an oncogenic miRNA in biliary tract cancer through down-regulating farnesoid X receptor expression, Gene, 493 (2012) 44-51.
[101] Q. Wang, H. Tang, S. Yin, C. Dong, Downregulation of microRNA-138 enhances the proliferation, migration and invasion of cholangiocarcinoma cells through the upregulation of RhoC/p-ERK/MMP-2/MMP-9, Oncol Rep, 29 (2013) 2046-2052.
[102] R. Yang, Y. Chen, C. Tang, H. Li, B. Wang, Q. Yan, J. Hu, S. Zou, MicroRNA-144 suppresses cholangiocarcinoma cell proliferation and invasion through targeting platelet activating factor acetylhydrolase isoform 1b, BMC Cancer, 14 (2014) 917.
[103] J. Iwaki, K. Kikuchi, Y. Mizuguchi, Y. Kawahigashi, H. Yoshida, E. Uchida, T. Takizawa, MiR-376c down-regulation accelerates EGF-dependent migration by targeting GRB2 in the HuCCT1 human intrahepatic cholangiocarcinoma cell line, PLoS One, 8 (2013) e69496.
[104] J. Li, F. Tian, D. Li, J. Chen, P. Jiang, S. Zheng, X. Li, S. Wang, MiR-605 represses PSMD10/Gankyrin and inhibits intrahepatic cholangiocarcinoma cell progression, FEBS Lett, 588 (2014) 3491-3500.
[105] A.V. Olaru, G. Ghiaur, S. Yamanaka, D. Luvsanjav, F. An, I. Popescu, S. Alexandrescu, S. Allen, T.M. Pawlik, M. Torbenson, C. Georgiades, L.R. Roberts, G.J. Gores, A. Ferguson-Smith, M.I. Almeida, G.A. Calin, E. Mezey, F.M. Selaru, MicroRNA down-regulated in human cholangiocarcinoma control cell cycle through multiple targets involved in the G1/S checkpoint, Hepatology, 54 (2011) 2089-2098.
[106] L. Yao, C. Han, K. Song, J. Zhang, K. Lim, T. Wu, Omega-3 Polyunsaturated Fatty Acids Upregulate 15-PGDH Expression in Cholangiocarcinoma Cells by Inhibiting miR-26a/b Expression, Cancer Res, 75 (2015) 1388-1398.
[107] J. Zhang, C. Han, T. Wu, MicroRNA-26a promotes cholangiocarcinoma growth by activating beta-catenin, Gastroenterology, 143 (2012) 246-256 e248.
[108] C. Hu, F. Huang, G. Deng, W. Nie, W. Huang, X. Zeng, miR-31 promotes oncogenesis in intrahepatic cholangiocarcinoma cells via the direct suppression of RASA1, Exp Ther Med, 6 (2013) 1265-1270.
[109] K. Okamoto, K. Miyoshi, Y. Murawaki, miR-29b, miR-205 and miR-221 enhance chemosensitivity to gemcitabine in HuH28 human cholangiocarcinoma cells, PLoS One, 8 (2013) e77623.
[110] H. Zhu, C. Han, D. Lu, T. Wu, miR-17-92 cluster promotes cholangiocarcinoma growth: evidence for PTEN as downstream target and IL-6/Stat3 as upstream activator, Am J Pathol, 184 (2014) 2828-2839.
[111] F. An, S. Yamanaka, S. Allen, L.R. Roberts, G.J. Gores, T.M. Pawlik, Q. Xie, M. Ishida, E. Mezey, A.C. Ferguson-Smith, Y. Mori, F.M. Selaru, Silencing of miR-370 in human cholangiocarcinoma by allelic loss and interleukin-6 induced maternal to paternal epigenotype switch, PLoS One, 7 (2012) e45606.

[112] J.L. Mott, S. Kobayashi, S.F. Bronk, G.J. Gores, mir-29 regulates Mcl-1 protein expression and apoptosis, Oncogene, 26 (2007) 6133-6140.
[113] N. Razumilava, S.F. Bronk, R.L. Smoot, C.D. Fingas, N.W. Werneburg, L.R. Roberts, J.L. Mott, miR-25 targets TNF-related apoptosis inducing ligand (TRAIL) death receptor-4 and promotes apoptosis resistance in cholangiocarcinoma, Hepatology, 55 (2012) 465-475.
[114] J. Zhang, C. Han, H. Zhu, K. Song, T. Wu, miR-101 inhibits cholangiocarcinoma angiogenesis through targeting vascular endothelial growth factor (VEGF), Am J Pathol, 182 (2013) 1629-1639.
[115] J. Li, B. Gao, Z. Huang, T. Duan, D. Li, S. Zhang, Y. Zhao, L. Liu, Q. Wang, Z. Chen, K. Cheng, Prognostic significance of microRNA-203 in cholangiocarcinoma, Int J Clin Exp Pathol, 8 (2015) 9512-9516.

**Figure 1**

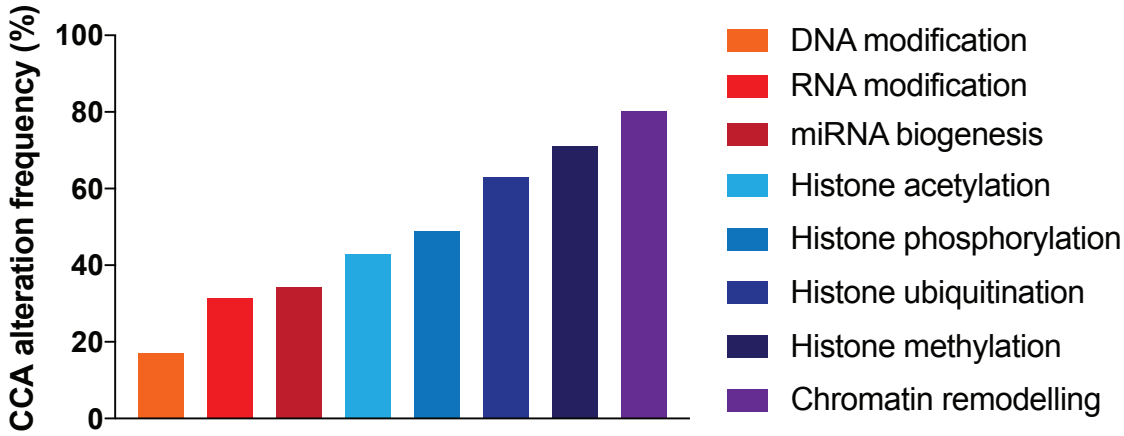
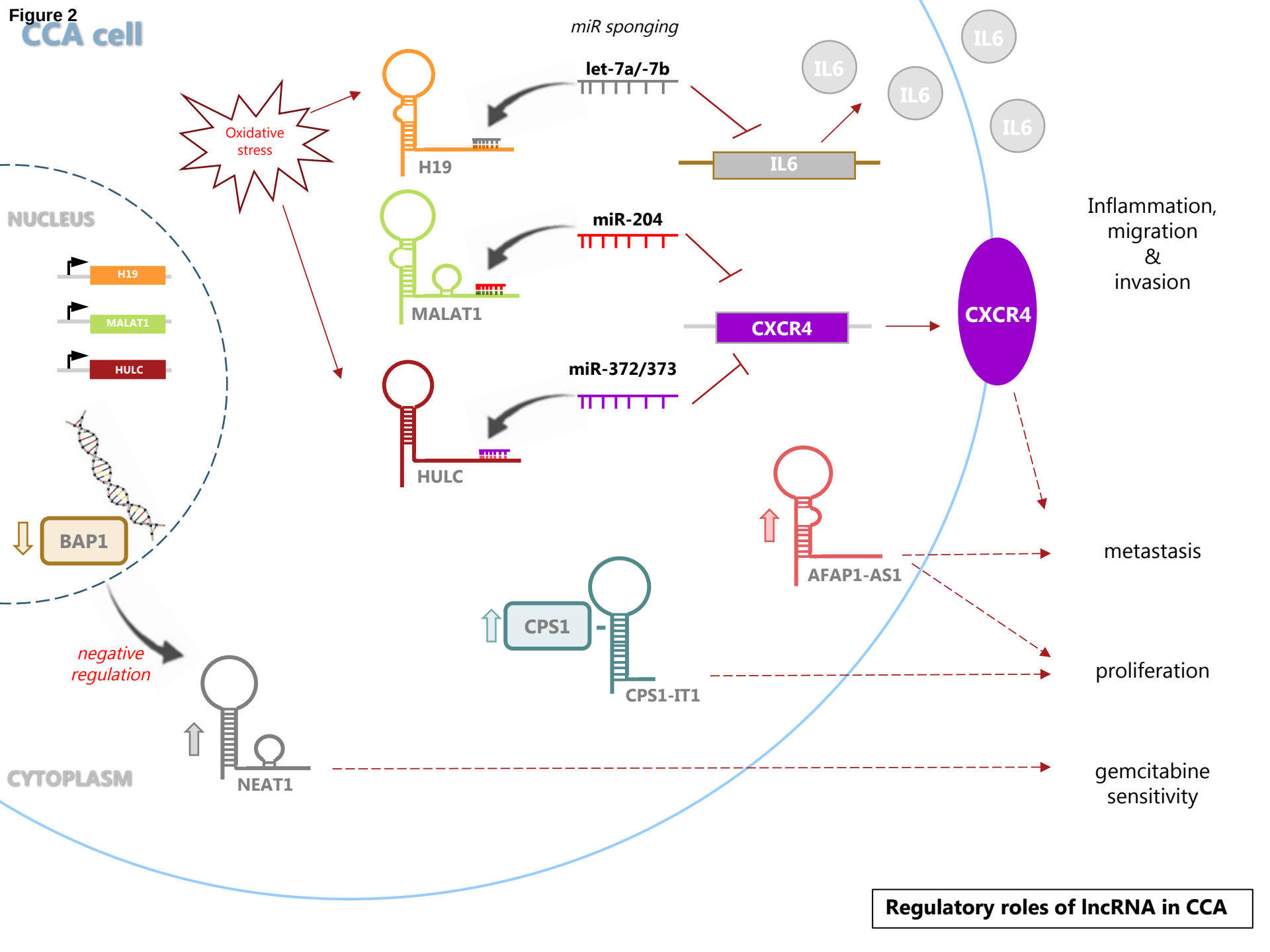

CCA alteration frequency (%)
100
80
60
40
20
0
DNA modification
RNA modification
miRNA biogenesis
Histone acetylation
Histone phosphorylation
Histone ubiquitination
Histone methylation
Chromatin remodelling

Figure 2




**Regulatory roles of lncRNA in CCA**

Figure 3

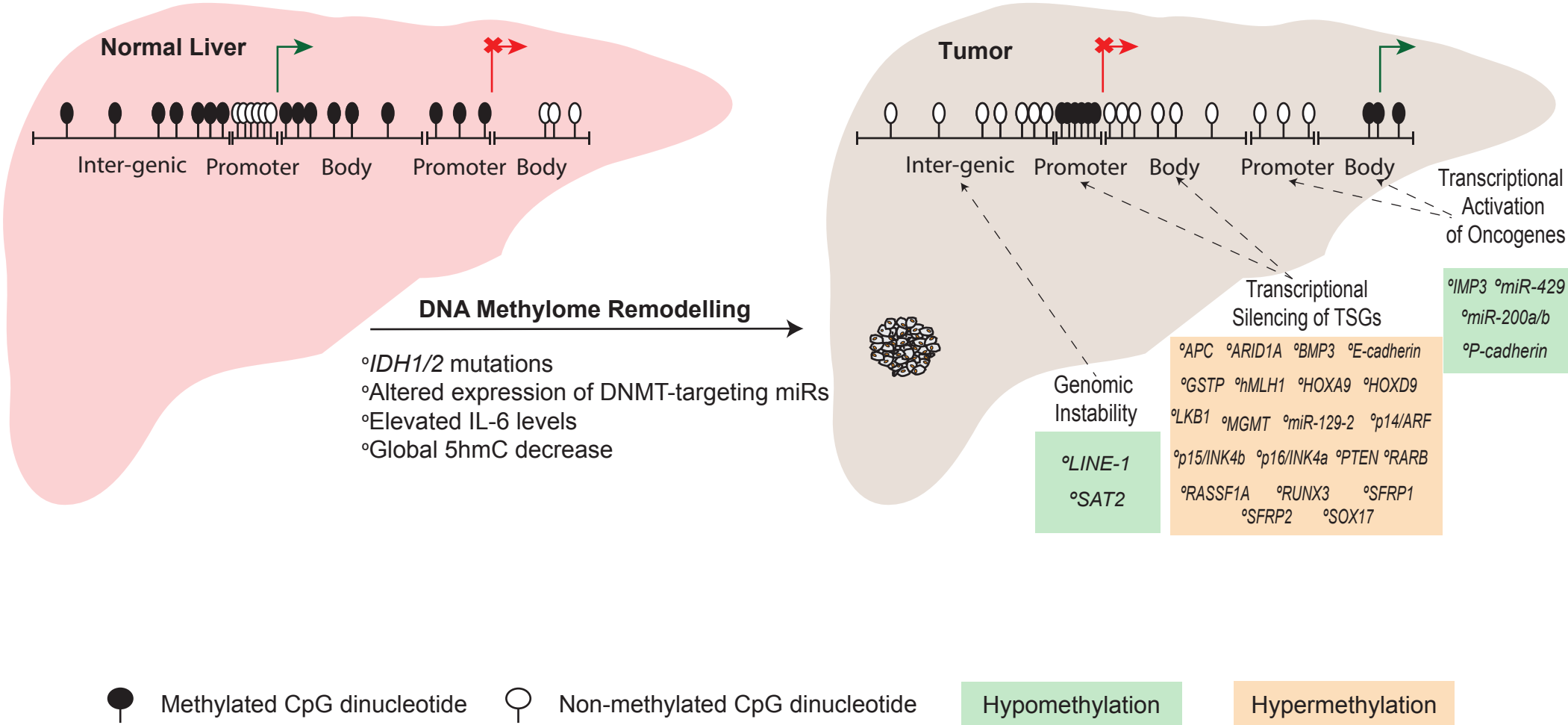

Normal Liver
Inter-genic
Promoter
Body
Promoter
Body
DNA Methylome Remodelling
°IDH1/2 mutations
°Altered expression of DNMT-targeting miRs
°Elevated IL-6 levels
°Global 5hmC decrease
Tumor
Inter-genic
Promoter
Body
Promoter
Body
Transcriptional Activation of Oncogenes
°IMP3 °miR-429
°miR-200a/b
°P-cadherin
Transcriptional Silencing of TSGs
°APC °ARID1A °BMP3 °E-cadherin
°GSTP °hMLH1 °HOXA9 °HOXD9
°LKB1 °MGMT °miR-129-2 °p14/ARF
°p15/INK4b °p16/INK4a °PTEN °RARB
°RASSF1A °RUNX3 °SFRP1
°SFRP2 °SOX17
Genomic Instability
°LINE-1
°SAT2
Methylated CpG dinucleotide
Non-methylated CpG dinucleotide
Hypomethylation
Hypermethylation

Figure 4

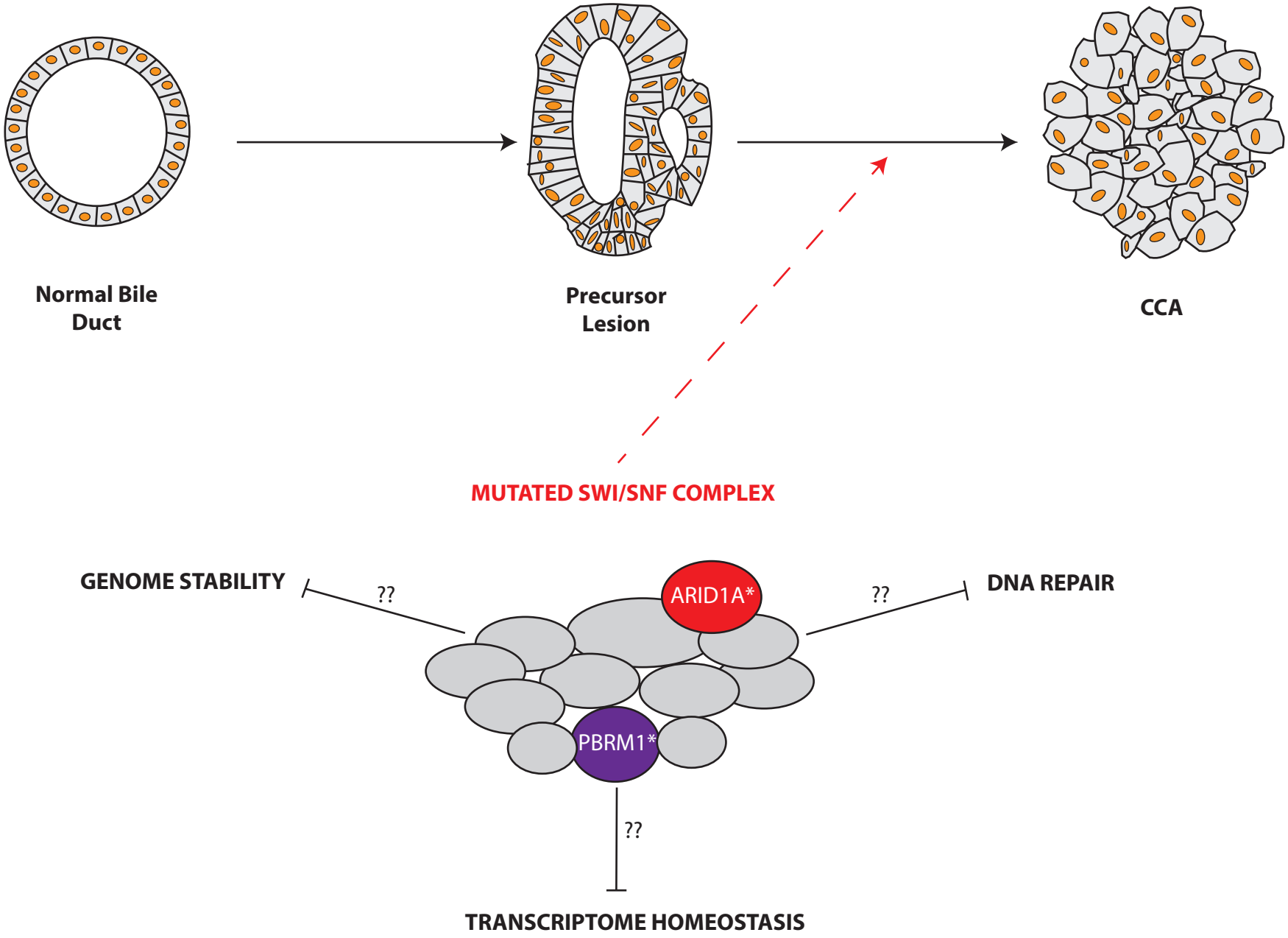

Normal Bile Duct
Precursor Lesion
CCA
MUTATED SWI/SNF COMPLEX
GENOME STABILITY
??
ARID1A*
??
DNA REPAIR
PBRM1*
??
TRANSCRIPTOME HOMEOSTASIS
ARID1A*
DUF3518
0
400
800
1200
1600
2000
2285aa
PBRM1*
Bro..
Bro..
Bro..
Bro..
Bro..
Bro..
BAH
BAH
0
400
800
1200
1689 aa

**Figure 5**

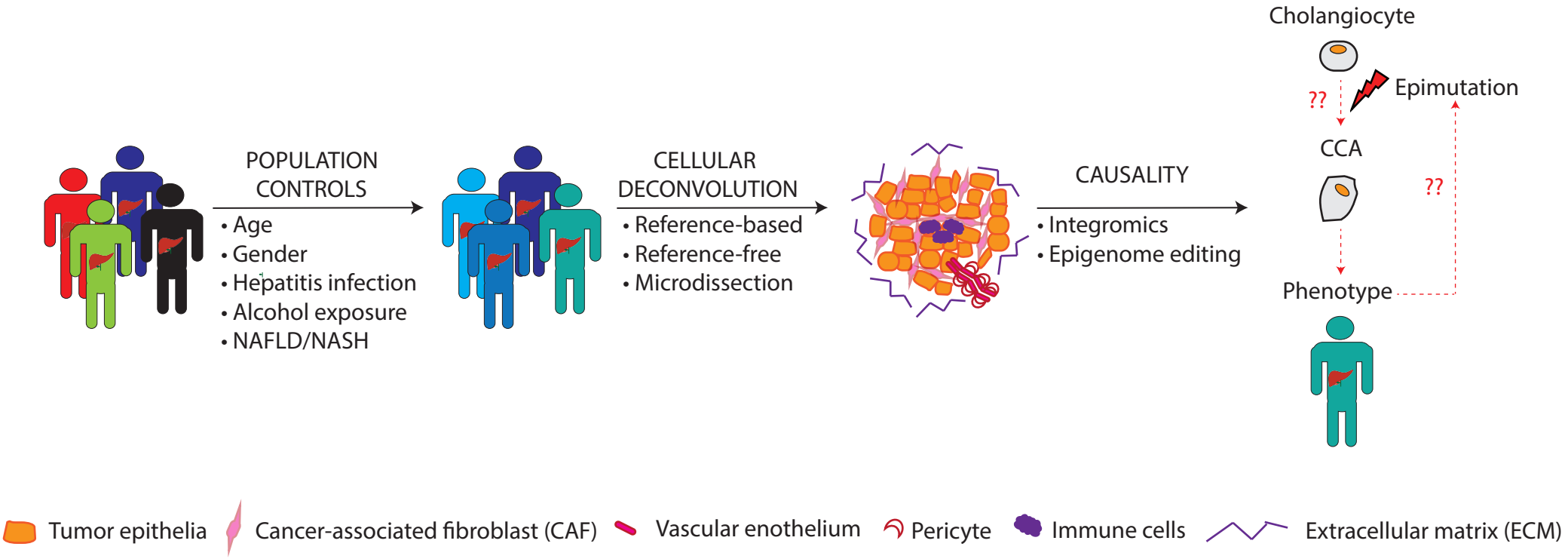

POPULATION CONTROLS
• Age
• Gender
• Hepatitis infection
• Alcohol exposure
• NAFLD/NASH
CELLULAR DECONVOLUTION
• Reference-based
• Reference-free
• Microdissection
CAUSALITY
• Integromics
• Epigenome editing
Cholangiocyte
??
Epimutation
CCA
??
Phenotype
Tumor epithelia
Cancer-associated fibroblast (CAF)
Vascular enothelium
Pericyte
Immune cells
Extracellular matrix (ECM)

**Table 1.** MicroRNA expression in CCA, target genes and functions.

| miR | expression | Target(s) | Role | Ref. |
|---|---|---|---|---|
| miR-21 | ↑ | RECK, PTEN, PTPN14 | Migration/invasion, proliferation | [18, 20, 99] |
| miR-421 | ↑ | FXR | Migration/invasion, proliferation | [100] |
| miR-124 | ↓ | SMYD3 | Migration/invasion | [59] |
| miR-138 | ↓ | RhoC | Migration/invasion, proliferation | [101] |
| miR-144 | ↓ | LIS1 | Migration/invasion, proliferation | [102] |
| miR-200b/c | ↓ | ROCK2; SUZ12 | Migration/invasion; drug resistance | [23] |
| miR-376c | ↓ | GRB2 | Migration | [103] |
| miR-605 | ↓ | PSMD10/Gankyrin | Migration/invasion, apoptosis, inflammation | [104] |
| miR-494 | ↓ | CDK6 | Proliferation | [105] |
| miR-26a/b | ↑ | 15-PGDH/HPGD | Proliferation | [106] |
| miR-141 | ↑ | CLOCK | Proliferation, cyrcadian rhythm | [18] |
| miR-200b | ↑ | PTPN12 | Proliferation, drug resistance | [18] |
| miR-26a | ↑ | GSK-3β | Proliferation, colony formation | [107] |
| miR-31 | ↑ | RASA1 | Proliferation, apoptosis resistance | [108] |
| miR-214 | ↓ | Twist | EMT | [27] |
| miR-200c | ↓ | NCAM1 | EMT | [22] |
| miR-34a | ↓ | SMAD4 | EMT | [24] |
| miR-204 | ↓ | SNAI2, Bcl2 | EMT | [25, 26] |
| miR-221 | ↑-↓ | PTEN; $PIK_3R1$ | EMT; drug resistance | [28, 109] |
| miR-17-92 cluster | ↑ | PTEN | EMT, migration/invasion, proliferation, cell cycle, inflammation | [110] |
| miR-let7a | ↑ | NF2 | Drug resitance & survival | [38] |
| miR-320 | ↓ | Mcl-1 | Drug resistance | [26] |
| miR-205 | ↓ | *To be clarified* | Drug resistance | [109] |
| miR-148a | ↓ | DNMT1 | Proliferation, DNA methylation, poor prognosis | [30] |
| miR-152 | ↓ | DNMT1 | Proliferation, DNA methylation, poor prognosis | [30] |

| | | | | |
|---|---|---|---|---|
| miR-370 | ↓ | MAP3K8; WNT10B | Proliferation, DNA methylation | [29, 111] |
| miR-373 | ↓ | MBD2 | Epigenetic changes in DNA methylation | [31, 32] |
| miR-29b | ↓ | Mcl-1; $PIK_3R1$, MMP-2 | Apoptosis; drug resistance | [109, 112] |
| miR-25 | ↑ | TRIAL DR4 | Apoptosis resistance | [113] |
| miR-410 | ↓ | XIAP | Apoptosis resistance | [34] |
| miR-101 | ↓ | COX-2, VEGF | Angiogenesis, tumor growth | [114] |
| miR-203 | ↓ | | Clinical relevance (poor prognosis) | [115] |

*Abbreviations: RhoC, Ras homolog gene family, member C; LIS1, platelet-activating factor acetylhydrolase isoform 1b; NCAM1, neural cell adhesion molecule 1; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; RASA1, RAS p21 GTPase activating protein 1; TRIAL DR4, TNF-realted apoptosis inducing ligand death receptor-4; ROCK2, Rho-associated protein kinase 2; XIAP, X-linked inhibitor of apoptosis protein; PTEN, Phosphatase and tensin homolog; RECK, Reversion-inducing-cysteine-rich protein with kazal motifs; PTPN14, Tyrosine-protein phosphatase non-receptor type 14; PTPN12, Tyrosine-protein phosphatase non-receptor type 12; SMAD4, SMAD family member 4; FXR, Farnesoid X receptor; SMYD3, SET and MYND domain-containing protein 3; SUZ12, Polycomb protein SUZ12; SNAI2, snail family transcriptional repressor 2; Bcl2, B-cell lymphoma 2; $PIK_3R1$, Phosphatidylinositol 3-kinase regulatory subunit alpha; GRB2, Growth factor receptor-bound protein 2; PSMD10, 26S proteasome non-ATPase regulatory subunit 10; GSK-3ß, Glycogen synthase kinase 3 beta; HPGD, Hydroxyprostaglandin dehydrogenase 15-(NAD); CLOCK, Circadian Locomotor Output Cycles Kaput; Mcl-1, Induced myeloid leukemia cell differentiation protein; MMP-2, Matrix metalloproteinase-2; COX-2, Prostaglandin-endoperoxide synthase 2; VEGF, Vascular endothelial growth factor; DNMT1, DNA (cytosine-5)-methyltransferase 1; MAP3K8, Mitogen-activated protein kinase kinase kinase 8; WNT10B, Wnt Family Member 10B; CDK6, Cell division protein kinase 6; MBD2, Methyl-CpG-binding domain protein 2; NF2, Neurofibromin 2.*

***Conflict of Interest Form**

**Click here to download Conflict of Interest Form: ORourke&Munoz-Garrido_etal_coi_disclosure.pdf**